# Regulation of *Staphylococcus aureus* infection of macrophages by CD44 and acid sphingomyelinase

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千里之行，始于足下。

**A thousand miles begins with a single step.**

## Index

**Abbreviation**

| | |
|---|---|
| methicillin-resistant *S. aureus* strains | MRSA |
| phosphorylated ERM | pERM |
| intercellular adhesion molecule 1 | ICAM-1 |
| sphingomyelin phosphodiesterase 1 | *Smpd1* |
| bone marrow-derived macrophages | BMDMs |
| alveolar macrophages | AMs |
| bronchoalveolar lavage | BAL |
| multiplicity of infection | MOI |
| colony-forming units | CFUs |
| tetramethylrhodamine isothiocyanate | TMR |
| fluorescein isothiocyanate | FITC |
| lysosome-associated membrane protein 1 | Lamp1 |

**Abstract**

*Staphylococcus aureus* plays an important role in sepsis, pneumonia, and wound infections. Acid sphingomyelinase (Asm) -deficient mice are highly susceptible to pulmonary *S. aureus* infections. We identified CD44 as a novel receptor for *S. aureus* in macrophages. CD44 activation by *S. aureus* stimulates Asm, resulting in ceramide release, clustering of CD44 in ceramide-enriched membrane platforms, a CD44/Asm-dependent activation of Rho family GTPases, a translocation of phospho-ezrin/radixin/moesin to the plasma-membrane, and a rapid rearrangement of the actin cytoskeleton with cortical actin polymerization. Genetic deficiency of CD44 or Asm abrogates these signaling events and thereby reduces internalization of *S. aureus* by macrophages by approximately 60% to 80%. Asm-deficient macrophages also exhibit reduced fusion of phagosomes with lysosomes, which prevents intracellular killing of *S. aureus* in macrophages and thereby allows internalized pathogen to replicate and cause severe pneumonia. Thus, the CD44-Asm-ceramide system plays an important role in the infection of macrophages with *S. aureus*.

# 1. Introduction

## 1.1 *Staphylococcus aureus (S. aureus)*

### 1.1.1 Epidemiology of *S. aureus*

*Staphylococcus aureus* is a very common commensal bacterium which is a serious cause of morbidity and mortality worldwide (Wertheim et al., 2005a). *S. aureus* has remained a leading cause of healthcare issue and financial cost over the past decades. External acquisition of *S. aureus* could be the initiation of an infection into an open wound. Frequently, the human host is colonized with the bacteria on the skin and mucosae (von Eiff et al., 2001; Wertheim et al., 2004). Remarkably, about 20% of populations are persistent nasal carrier of *S. aureus* and approximately 30% are intermittent colonized (Eriksen et al., 1995; Hu et al., 1995; Kluytmans et al., 1997; Nouwen et al., 2004). Persistent nasal carriage is more frequent in children than adults (Armstrong-Esther, 1976), particularly, more than 70% of infants have been determined with at least one strain of *S.aureus* (Peacock et al., 2003).Extra-nasal infection of *S. aureus* includes the skin, perineum, pharynx (Ridley, 1959; Wertheim et al., 2005b; Williams, 1963). Other sites including gastrointestinal tract, vaginal wall, and axillae are less frequently colonized (Dancer and Noble, 1991; Guinan et al., 1982; Williams, 1963).

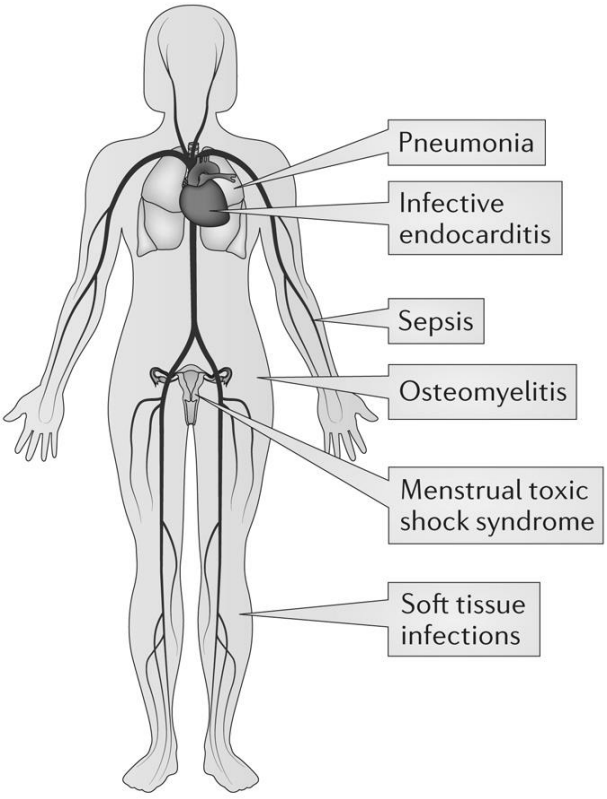



Figure 1.1.1 Large diversities in *S.aureus* infection. *S. aureus* causes minor skin infection to life-threatening diseases (Salgado-Pabon and Schlievert, 2014).

*S. aureus* is one of the most important causes of a broad array of clinical infections (Fig. 1.1.1) (Salgado-Pabon and Schlievert, 2014). Skin and soft tissue infection such as boils, folliculitis, impetigo and cellulitis are

the most frequent illness form of the bacteria, and the progression these infections can lead to bacteremia and severe invasive disease, including bloodstream infection, endocarditis or sepsis (David and Daum, 2010; Lowy, 1998; Talan et al., 2011). In addition, the organism can cause osteomyelitis, infectious arthritis, abscesses in many organ tissues, toxic-shock syndrome, surgical-site and prosthetic materials infections, and pneumonia (DeLeo et al., 2010; Fridkin et al., 2005; Klevens et al., 2007). The prevalence and incidence of *S. aureus* diseases range between 1-3% depends on age, race, and geographical location (David and Daum, 2010). The risk of *S. aureus* diseases elevates in early-born infants, children, elderly, and patients with immunosuppression, diabetes, and dialysis (Kluytmans et al., 1997). The recurrence is a key characteristic of the skin, soft tissue and blood infection, is found for 8-33% of all patients (Kallen et al., 2010). Many *S. aureus* strains are resistant to antibiotics (Corey, 2009; Grundmann et al., 2006; Maskalyk, 2002); in particular, methicillin-resistant *S. aureus* strains (MRSA) have become an important clinical problem and are now recognized as serious pathogens in communities and hospitals worldwide (Marimuthu and Harbarth, 2014; Peyrani and Ramirez, 2015; Singer and Talan, 2014).

*S. aureus* plays a predominant role in hospital-acquired infections: hospital-acquired pneumonia, ventilator-associated pneumonia, and health care-associated pneumonia (American Thoracic and Infectious Diseases Society of, 2005). Overall, *S. aureus* has been implicated as one of the most common pathogen which accounts for more than 40% culture-positive health care-associated pneumonia cases (Kollef et al., 2005). Recently, a distinct strain of *S. aureus* named USA 400, which is encoded with genes for Panton-Valentine leukocidin (PVL), has emerged and causes a severe necrotizing pneumonia (1999; Gillet et al., 2002). PVL is a member of the family of bicomponent β-channel pore-forming toxins targeting phagocytic leukocytes in *S. aureus* necrotizing infections (Diep et al., 2010; Loffler et al., 2010). The success of *S. aureus* acting as a respiratory pathogen is in virtue of several features: substantial metabolism, genetic flexibility that acquires and mutates specific genetic elements, and the capability of exploiting the evoked immune responses (al-Ujayli et al., 1995; Parker and Prince, 2012). *S. aureus* is regularly recognized as extracellular aerobic pathogen; however the organism also

survives and replicates intracellular (da Silva et al., 2004; Kapral and Shayegani, 1959) and tolerates anaerobic conditions (Belay and Rasooly, 2002) which are connected to pulmonary infections.

### 1.1.2 Components and Products of *S. aurues*

*S. aureus* is a gram-positive bacterium belonging to the Micrococcaceae family (Fig. 1.1.2) (Lowy, 1998). *S. aureus* is discriminated from other staphylococcal species on the appearance of the gold-yellow colonies, which are positive for coagulase, mannitolfermentation, and deoxyribonuclease tests.

The genome of *S. aureus* consists of a circular chromosome of approximately 2.7–2.8 Mbp plus an assortment of extrachromosomal accessory gene elements: prophages, plasmids, mobile elements, other variable elements (Baba et al., 2008; Mlynarczyk et al., 1998). Genes carrying virulence and antibiotic resistance determinants that induce the development of clinical diseases was found on chromosome and extrachromosomal elements (Baba et al., 2002; Novick, 1991).

The staphylococcal cell wall is constituted of peptidoglycan, teichoic acids, and proteins (Giesbrecht et al., 1976; Umeda et al., 1987). Over 70% of the cell wall is peptidoglycan by weight and teichoic acid binds to the peptidoglycan through a phosphodiester bond (Heptinstall et al., 1978). These polymers in the cell wall arranges circularly where the bacteriophage receptors on the bacterial surfaces are located (Umeda et al., 1980). The peptidoglycan chains are linked by pentaglycine cross-bridge and by tetrapeptide chains bound to N-acetylmuramic acid (Ton-That et al., 1997).Variation in the peptidoglycan structure is response to the differences in their ability to cause disseminated intravascular coagulation (DIC) (Kessler et al., 1991).

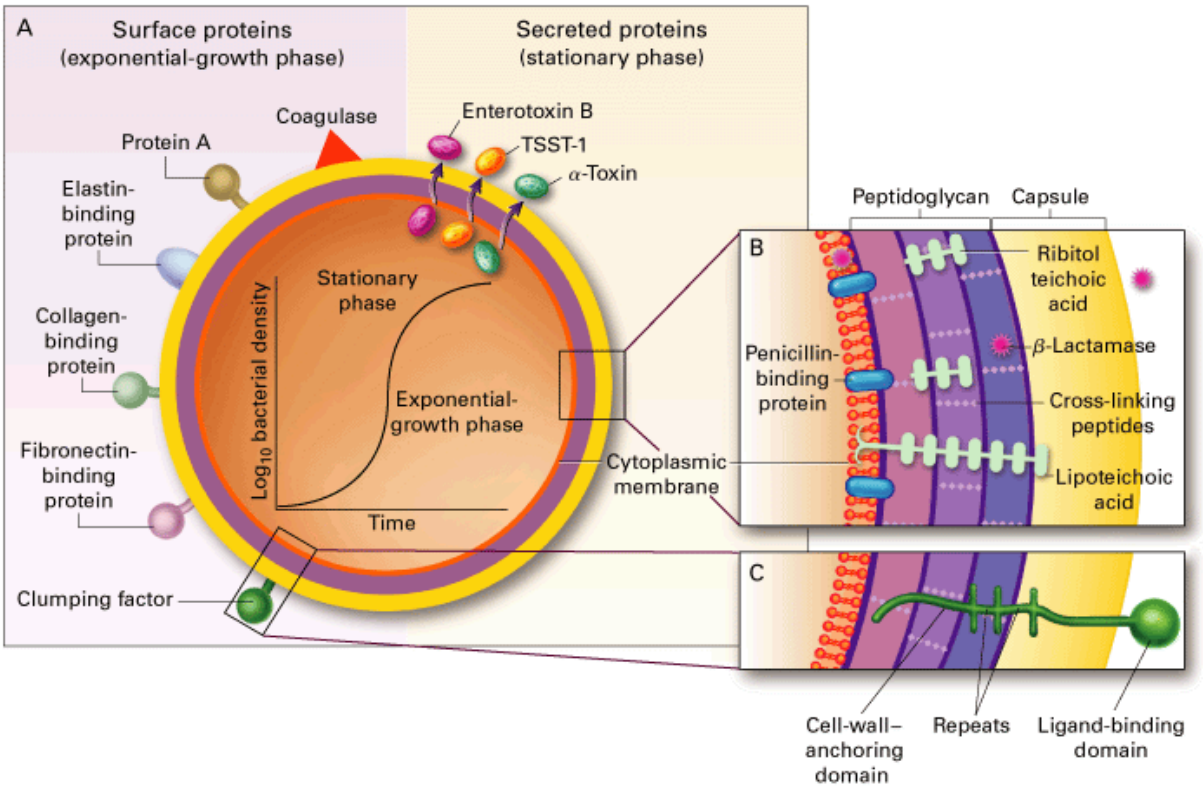



Figure 1.1.2 Structure of *S. aureus* (Lowy, 1998).

Panel A: The surface and secreted proteins. The synthesis of these proteins is dependent on the growth phase and is controlled by regulatory genes such as agr. Panels B and C: Cross sections of the cell envelope. Many of the surface proteins and peptides have a similar structural organization as clumping factor, including repeated segments of amino acids. TSST-1 denotes toxic shock syndrome toxin 1.

Capsule of *S. aureus* was first reported in 1931 (Gilbert, 1931). At least 18 strains of capsule of *S. aureus* have been described and partially characterized (O'Riordan and Lee, 2004). Most clinical isolates of *S. aureus* produces type 5 or type 8, which accounts for 75% of human infections. The capsule impedes the phagocytosis therefore enhancing virulence of the organism, finally the bacterial persistence in hosts (Thakker et al., 1998).

Staphylococcal surface proteins are covalently attached to peptidoglycan. The structure of these proteins is composed of a signal sequence at the N terminal which is cleaved during secretion, and a wall-spanning region and sorting signal at the carboxyl terminal facilitating the covalent anchorage to peptidoglycan (Foster et al., 2014). Ligand-binding

domain at the N terminal on the bacterial surface functions as adhesins of some proteins (Foster and McDevitt, 1994). Protein A has an ability binds to the Fc region of immunoglobulin resulting antiphagocytic properties (Fig. 1.1.2). In addition, protein A mediates the activation of host intracellular signaling and increase of inflammation, leading severe pneumonia (Normark et al., 2004; Soong et al., 2011). Several surface proteins bind extracellular-matrix molecules and function in invasion of host cells and evasion of elimination of host cells, of which microbial surface component recognizing adhesive matrix molecules (MSCRAMMs) are the largest class (Patti et al., 1994).

*S. aureus* produces numerous toxins which are classified into three families: pore-forming toxins, exfoliative toxins and superantigens (Grumann et al., 2014). Pore-forming toxins destruct the membranes of host cells, eventually induce the cell lysis. These pore-forming toxins potentially function as cell stressors at sublytic concentrations, synergically effect with signals such as lipoproteins and activating toll-like receptor 2 (TLR2) and NALP3-inflammasomes, finally the release of cytokines (Franchi et al., 2012). Hemolysin-α (Hla, α-toxin), Panton-Valentine leukocidin (PVL) and hemolysin-γ (Hlg) have been shown strongly evoking the inflammatory responses (Holzinger et al., 2012; Kebaier et al., 2012; Perret et al., 2012). Exfoliative toxins are functionally isoforms of enzymes with high species-specificity. Exfoliative toxins have glutamate-specific serine protease activity and cause skin erythema and separation as “molecular scissors” (Nishifuji et al., 2008). The staphylococcal superantigens are considered as enterotoxins since they induce food poisoning syndrome such as vomiting and diarrhea after oral uptake (Harris et al., 1993). In addition, the superantigens interact with major histocompatibility complex (MHC) class II proteins by binding to the α-chain or to a conserved histidine in the β-domain, and trigger extensive T-cell proliferation and cytokine release (Fraser and Proft, 2008; Marrack and Kappler, 1990).

*S. aureus* produce various enzymes degrading molecules, interfering metabolic or signaling cascades of hosts. Several enzymes function as proteases which non-specifically or specifically degrade host proteins and leads to tissue damage. The protease aureolysin cleaves proteins with a preference of cleaving after hydrophobic

residues (Laarman et al., 2011). It can also inactivate phenol-soluble modulins thus triggering osteoblast cell death and bone destruction (Cassat et al., 2013). In addition, Aureolysin, glutamyl endopeptidase, and the cysteine proteases staphopain appear to be interfering with complement factors, leading to pathogen-mediated evasion of the human complement system (Jusko et al., 2014). The biological function of a series of *S. aureus* serine proteases is not well defined. Staphylokinase is known to convert plasminogen into plasmin which mediates fibrinolysis. This biological effect is to disrupt the function of fibrin meshwork thus localizing staphylococcal infection (Okada et al., 2000). Further staphylokinase assists invasion of organism through the skin barrier (Kwiecinski et al., 2013). Two coagulases, staphylocoagulase and von Willebrand factor (vWF), mediates the activation of host prothrombin and formation of fibrin cables, thus facilitating *S. aureus* clot formation and establishment of infectious (Thomer et al., 2013). *S. aureus* produces nucleases, which may decrease the bactericidal capacity of neutrophil extracellular traps (NET), which consist of rocessed chromatin bound to granular and selected cytoplasmic proteins (Brinkmann and Zychlinsky, 2012). The function of staphylococcal lipases is not well understood, presumably these enzyme degrade triglycerides to release free fatty acids which involve in the bacteria growth. Importantly, *S. aureus* beta-toxin is a sphingomyelinase that hydrolyzes sphingomyelin to yield ceramide and phosphorylcholine, a key enzyme will be discussed in our study. The beta-toxin is a critical virulent factor contributing significantly to the pathogenesis of *S. aureus*, including lung injury (Hayashida et al., 2009).

### 1.1.3 Pathogenesis of *S. aureus*

*S. aureus* virulence genes are thought to be unregulated after exposure to the mucosal surface or skin of host (Novick, 2003). Bacterial products or signals from host immune response activate the resident phagocytes and epithelial cells in the localized skin or mucosal tissue. Host molecules such as TLR2 recognize staphylococcal peptidoglycan and lipoprotein (Fournier, 2012; Hashimoto et al., 2006); furthermore, hyaluronan (HA), a ubiquitously distributed extracellular matrix ligand for CD44, breakdowns into lower molecular weight fragments which can further facilitate pro-inflammatory signaling

resulting local immune cell activation, and recruitment of neutrophil and macrophage (Scheibner et al., 2006).

*S. aureus* has been generally recognized surviving both extracellular and intracellular organism of host cells. *S. aureus* escapes from opsonization by complement and antibodies which target the bacteria for uptake or elimination by phagocytes through Fc or complement receptors. This pathogen evades opsonophagocytosis by expressing on its surface a capsule, protein A, and variety of complement inhibitors, all of which serves to blocking and avoiding host opsonins from targeting the organism for destruction (Foster, 2005; Rooijakkers et al., 2005).

*S. aureus* can survive within a variety of mammalian epithelial cells, endothelial cells, non-professional phagocytes, and professional phagocytes such as neutrophils and monocytes (Kubica et al., 2008). *S. aureus* employ several strategies to avoid formidable challenge by neutrophils. First, chemotaxis inhibitory protein (CHIP) and extracellular adherence protein (Eap) secreted by *S. aureus* abolish the recognition of chemotactic factors from neutrophils (de Haas et al., 2004), as well as interact with host adhesive proteins intercellular adhesion molecule 1 (ICAM-1) thus blocking neutrophil adhesion (Chavakis et al., 2002). Neutrophils release a series of products which serve to clear *S. aureus*, including antimicrobial peptides, reactive oxygen species (ROS), reactive nitrogen species (RNS), proteases, and lysozyme. However, *S. aureus* defense against ROS by secreting antioxidant enzymes, such as catalase, pigment, and superoxide dismutase which counteract ROS and RNS (Foster, 2005). *S. aureus* can also modify its own negatively charged bacteria surface thereby escaping targeting of antimicrobial peptides (Collins et al., 2002; Peschel et al., 2001). In addition, aureolysin (Sieprawska-Lupa et al., 2004) or staphylokinase (Jin et al., 2004) can degrade or neutralize antimicrobial peptides, respectively. Further, *S. aureus* produces a large amount of two-component toxins which lead to the neutrophils lysis (Tomita and Kamio, 1997). Recently phenol soluble modulin (PSM) has emerged as a novel toxin family of aggressive *S. aureus* isolates which highly induce inflammation and neutrophil cytolysis (Peschel and Otto, 2013).

Iron is vital for the survival and proliferation of *S. aureus* within the human during infection (Hammer and Skaar, 2011). Most iron is employed as a cofactor in intracellular biochemical reactions, and free iron within the serum is almost always bound to high-affinity iron binding proteins. In response of the iron acquisition, *S. aureus* has developed complex strategies to obtain iron needed to proliferate within hosts. Heme, representing most amount of iron within the host, is a important iron source of *S. aureus* (Skaar et al., 2004). During iron starvation, *S. aureus* dramatically change its protein expression which is mediated by the iron-dependent ferric uptake regulator (fur) (Friedman et al., 2006; Torres et al., 2006). Small molecules siderophores secreted by *S. aureus* have remarkably high affinity to iron. Two distinct siderophores produced by *S. aureus* named staphyloferrin A and staphyloferrin B are maximally synthesized in iron-limiting environments for bacterial survival (Friedman et al., 2006; Lindsay and Riley, 1994).

The adaptive immune system consists of highly specific, systemic cells and bioprocesses that limit the ongoing pathogen infection and prevent future re-infections. However, one feature of *S. aureus* pathogenesis is the capacity of infecting human host repeatedly throughout life. Studies have revealed that staphylococcal extracellular adherence protein, toxic shock syndrome toxin, and enterotoxins could impede T cell functions by blocking T cell receptor activation (Lee et al., 2002; Llewelyn and Cohen, 2002), which is the strategy of host developing long time memory of protect against *S. aureus* infection. Similarly, protein A induced preferential and prolonged deletion of innate-like B lymphocytes which are precursors of B cells (Goodyear and Silverman, 2004). Manipulation of B cell and T cell responses, together with strategies described above, could be possible underlying reasons that *S. aureus* infect throughout host lives.

The generation of biofilm by *S. aureus* plays a critical role in the persistence of chronic infections. The *S. aureus* biofilm matrix is composed of host factors, secreted and lysis-derived proteins, polysaccharide, and eDNA (Lister and Horswill, 2014). Individual bacterial can spread out from the original biofilm and develop new sites of infections or mediate sepsis (Costerton et al., 1999). The biofilm dispersal mechanism can be regulated by protease (Bronner et al., 2004; O'Neill et al., 2008), nuclease (Hernandez

et al., 2014; Olson et al., 2013), and dispersin-B (Kaplan et al., 2004). Other virulence mechanisms including small colony variants are linked to chronic, recurrent, and antibiotic-resistant infections (Proctor et al., 2014). Changes in small colony variants are found, including stringent response (Gao et al., 2010), RNAIII metabolism (Kohler et al., 2008), toxin-antitoxin,(Donegan and Cheung, 2009), and ribosomes (Lannergard et al., 2011), all of these alterations are responsible for bacterial survival within the harsh host environment.

### 1.1.4 *S. aureus* in pulmonary infection

Adaption of *S. aureus* to the environment of respiratory tract has facilitated its emergence as a respiratory pathogen. Serious respiratory syndrome has been apparently increasing due to emerge of more virulent USA300 community acquired MRSA strains (Klevens et al., 2007; Montgomery et al., 2008).

Accessory gene regulator (Agr), a regulatory system in the genome of *S. aureus*, coordinates the expression of both surface proteins and secreted toxins (Recsei et al., 1986). Agr mutations are also found in clinical strains (Traber et al., 2008). The sensor histidine kinase (AgrC) and the response regulator (AgrA) are necessary for the coordination of invasive infection of the lungs (Heyer et al., 2002), as well in the animal models of pneumonia (Bubeck Wardenburg et al., 2007; Montgomery et al., 2010). Additionally, Agr plays an important role in pathogenesis of intracellular circle of *S. aureus*. Agr is necessary for these intracellular bacteria escaping from endosomes and contributing to pulmonary damage (Qazi et al., 2001; Shompole et al., 2003). *S. aureus* has been observed in non-phagocytic cells such as some epithelial cells lines (Bayles et al., 1998; Qazi et al., 2001) and is linked to the cell apoptosis (da Silva et al., 2004). In phagocytic cells macrophages, *S. aureus* is able to persist and survive intracellular several days without affecting the viability of these mobile cells, until the pathogens escape into the cytoplasm and induce the cell lysis (Kubica et al., 2008). Likewise, studies has shown small number of *S. aureus* survive prolonged periods of time within neutrophils and occasionally multiply within dying cells (Melly et al., 1960), a

mechanism which effect to a systemic dissemination of the pathogen (Gresham et al., 2000).

As mentioned above, iron is essential for bacterial survival. Ferric uptake regulator (Fur), a homologue of the iron regulatory protein secreted by *S. aureus*, alters the abundance of a large number of virulence factors including α-hemolysin and PVL thus protecting *S. aureus* against killing by neutrophils in the pathogenesis of pneumonia (Torres et al., 2010). By coordinating the reciprocal expression of cytolysins and a subset of immunomodulatory proteins, Fur exhibit an impact on regulating the expression of virulent factors.

Varieties of surface proteins specifically recognize host cell and tissues, such as collagen, fibrinogen, and fibronectin. Epithelial cell damage permits the binding of Panton-Valentine leukocidin-positive *S. aureus* to exposed collagens and laminin during severe lung injury (de Bentzmann et al., 2004). Fibronectin-binding protein plays a major role in the colonization of human airway epithelial cells in staphylococcal infectious process (Mongodin et al., 2002). Clumping factors A and B mediates the adherence of *S. aureus* to fibrinogen (Higgins et al., 2006; Palmqvist et al., 2004). Clumping factor B promotes adherence to human type I cytokeratin 10 on nasal epithelial cells (O'Brien et al., 2002). Further studies confirms Clumping factor B is a major determinant of nasal *S. aureus* colonization (Wertheim et al., 2008).

The secretion of various toxins also contributes to pathogenesis of *S. aureus*. The α-toxin is a major pore-forming toxin of *S. aureus*. Expression of α-toxin is increased, associated with calcium fluxes and pro-inflammatory response (Yun et al., 1999), and change of ciliary beat frequency (Rose et al., 2002) in epithelial cells during infection. Recently, studies have shown that α-toxin binds to a disintegrin and metalloprotease 10 (ADAM10) with high-affinity, which is critical for causing cytotoxicity at low toxin concentrations (Wilke and Bubeck Wardenburg, 2010). ADAM10 is crucial for bacterial virulence and regulate acute and asthmatic inflammatory responses in lung injury (Dreymueller et al., 2015). α-toxin is involved in activating pyroptosis, inducing inflammasome via caspase-1 activation, targeting pro-IL-1β, and generation of IL-1β

(Parker and Prince, 2012). Consistently, studies using mutants of α-toxin have shown connections between α-toxin expression and virulence in the lung (Bubeck Wardenburg and Schneewind, 2008). Development of antibodies against α-toxin identifies further therapeutic opportunities in preventing lung injuries (Ragle and Bubeck Wardenburg, 2009; Ragle et al., 2010). Furthermore, β-toxin of *S. aureus* also contributes to the pulmonary infection. In airway epithelial cells β-toxin has been shown reduce ciliary activity (Kim et al., 2000). β-toxin is also associated with the increasing of airway permeability as well as a neutrophilic response in lung pathology (Hayashida et al., 2009). Panton–Valentine leukocidin (PVL) is a toxin encoded by lukF-PV and lukS-PV (Prevost et al., 1995), and forms octomeric protein pores at the cell membranes (Diep et al., 2010), all of which contributes the inflammations in the lung.

Protein A is an abundant surface protein which may be one of the most sophisticated staphylococcal components since its various interactions with host signaling including lung pathogenesis. Infection of protein A mutants significantly reduce the mortality of mice with pneumonia compared with the wild type (Bubeck Wardenburg et al., 2007). Protein A binds the Fab portion of V(H)3-type B cell receptors thus impedes the adaptive immune response (Hakoda et al., 1994). Tumor-necrosis factor-alpha receptor (TNFR1), a protein A receptor widely expressed on the airway epithelium, is activated and recruits polymorphonuclear leukocytes finally inducing pneumonia (Gomez et al., 2004). Additionally, protein A ubiquitously and multifunctionally stimulates EGFR and ERK phosphorylation to regulate TNFR1 on mucosal cells (Gomez et al., 2007). Moreover, EGFR activation stimulates the increased wound closure and transepithelial resistance, which results from increased proliferation and survival of uninjured epithelial cells (Shaykhiev et al., 2008).

In summary, *S. aureus* has deployed a series of mechanism surviving as a respiratory pathogen which not only success in persistence in the respiratory tract but also prosper as an invasive pulmonary pathogen. To prevent infection, multiples strategies should be considered, including virulence determinants, iron acquisition, biofilm production and immune evasion.

## 1.2 Acid sphingomyelinase and ceramide system

### 1.2.1 Lipid rafts

The fluid mosaic model, first introduced by Singer and Nicolson in 1972, is the major fundamental of our understanding of the structure of biological membranes (Singer and Nicolson, 1972). The theory is proposed as a basic framework model for cell membranes which could interprets studies on membrane proteins and structure and dynamics of lipids at the time. As originally described, the fluid mosaic model defined biological membranes as a matrix composed of fluid bilayer of phospholipids with mobile globular integral membrane proteins and glycoproteins that were intercalated into the fluid lipid bilayer, which is “protein icebergs floating in the sea of lipid” (Fig 1.2.1 A) (Nicolson, 2014; Singer and Nicolson, 1972). However, considering numerous later studies on membranes those have been published, our view of biological membrane structure has been extended in these years. Studies have demonstrated that lipids are not uniformly distributed in the cell membrane, and the membrane microdomain ‘lipid rafts’ was proposed (Lisanti et al., 1988; van Meer et al., 1987). Biochemically, lipids are sorted within the cell (van Meer et al., 2008). Lipid rafts are defined as self-associative properties of sphingolipid and cholesterol which promotes selective lateral segregation in the membrane plane and serves as a basis for lipid sorting (Simons and Ikonen, 1997). Recently, the new concept is established in the consensus description of lipid rafts at the Keystone Symposium of Lipid Rafts and Cell Function: Lipid rafts are small (10–200 nm), heterogeneous,

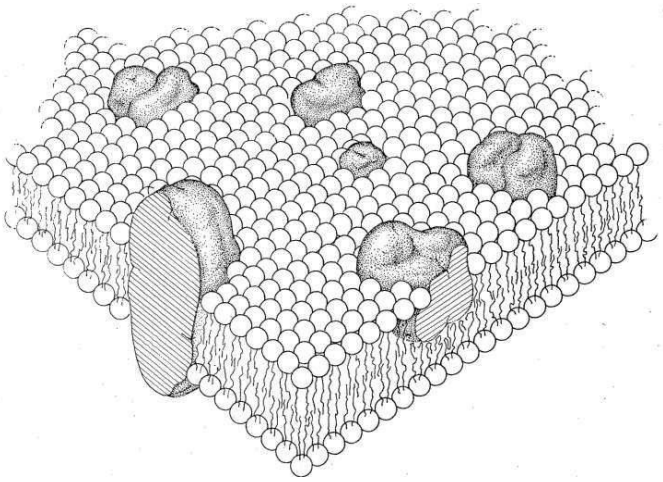


Figure 1.2.1 A. The fluid mosaic membrane model. In this cross-sectional structural view of a cell membrane the solid bodies with stippled cut surfaces represent globular integral membrane proteins, which at intermediate range are randomly distributed in the plane of the membrane. At short range, some integral membrane proteins form specific integral protein complexes (Nicolson, 2014).

highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein-protein and protein-lipid interactions (Pike, 2006). The studies on lipid rafts is continuous developing, Fig. 1.2.1 B lists the timeline of pioneer studies in the lipid raft field (Varshney et al., 2016).

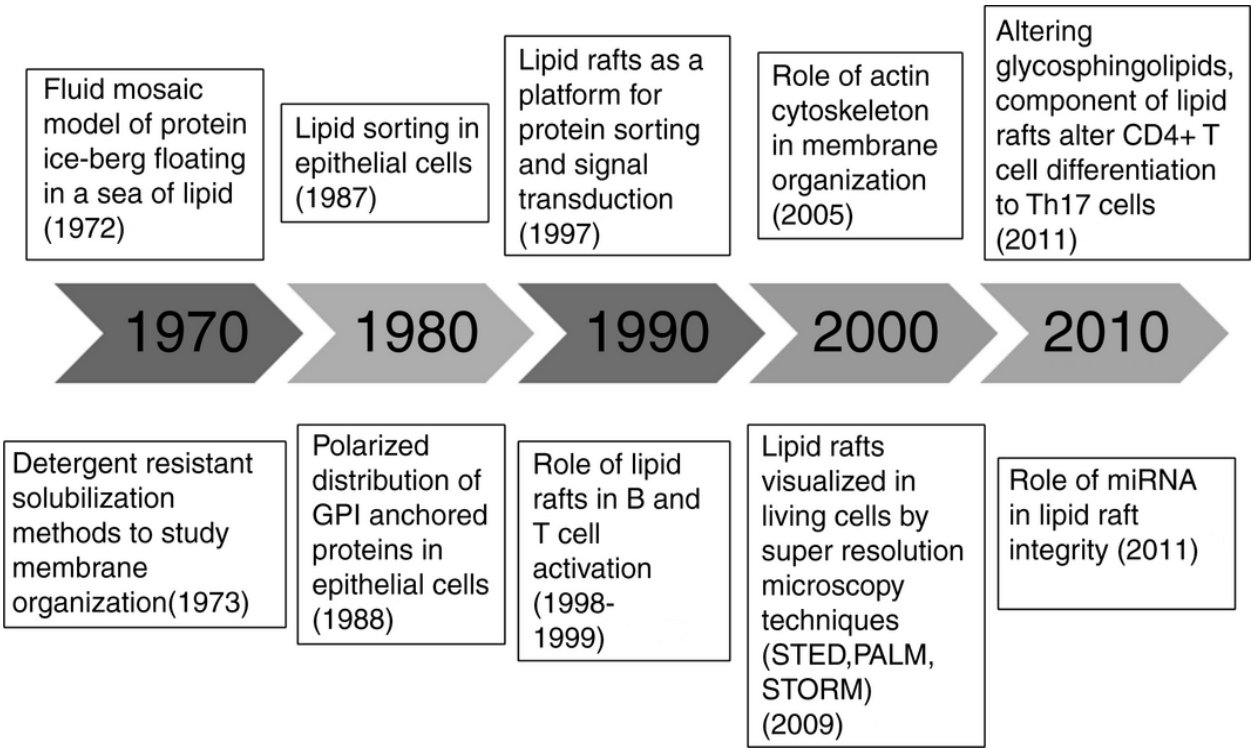



Figure 1.2.1 B. Timeline of pioneer discoveries in the field of lipid rafts and signaling.

Lipid rafts incorporate with multiple distinct classes of proteins: true resident proteins such as glycosylphosphatidylinositol (GPI)-linked proteins and caveolin (Brown and London, 1998; Hooper, 1999). signalling proteins such as doubly acylated proteins like Src family kinases, G-protein-coupled receptor (GPCR) proteins (Resh, 1999), cholesterol-linked and palmitoylated proteins such as hedgehog and myristoylated proteins (Brown and London, 1998; Rietveld et al., 1999). The mechanism that GPI-anchored or hydrophobic modifications carried proteins possibly due to preferential packing of their saturated membrane anchors into rafts. The kinetics or partition coefficients of proteins associating with rafts can be distinct. For example, a monomeric

transmembrane protein residents most of time outside the rafts, but when crosslinked or oligomerized, its affinity to rafts increased and stay longer in rafts (Harder et al., 1998). Further, clustering of separate rafts recruits proteins to a new membrane environment and initiates signaling cascades through amplification. These evidences, which lipids driven by lipid–lipid, lipid–protein and protein–protein interactions, are crucial for the activation of many signal transduction pathways.

Sphingolipids, cholesterol and (glycero)phospholipids are predominantly components of cell membranes. Particularly, sphingolipids and cholesterol seem to be not randomly distributed in the membrane. The interactions between these lipids result in spontaneous formation of distinct sphingolipid- and cholesterol-enriched very small membrane domains, termed rafts. Sphingolipids consist of a hydrophilic head group and a hydrophobic ceramide molecule. Sphingolipids, particularly the most abundant sphingolipid sphingomyelin, have a higher melting temperature compared with phospholipids in the cell membrane; tend to interact with each other through hydrophilic interactions of the hydrophilic sphingolipid head groups (Brown and London, 1998; Harder and Simons, 1997; Simons and Ikonen, 1997; Xu et al., 2001). Cholesterol functions as a spacer between the bulky head groups of sphingolipids head, coordinate with sphingolipids via hydrogen bonds and hydrophobic van der Waal interactions of the sterol ring system and the ceramide moiety of sphingolipids, thus interactions formed. The tight interactions induced stable domains exist in a liquid-ordered- or even gel-like phase, which are separated from other phospholipids in the cell membrane. Studies support the existence of these domains that cell membrane contains domains resistant to solubilization by non-ionic detergents (Brown and Rose, 1992). Powerful microscopy evidence further records the existence of lipid nanodomains rafts with a diameter of 20 nm in living cells (Eggeling et al., 2009). It should be noted that lipid rafts may exist in the outer leaflet of the cell membrane, while the existence of lipid rafts or similar membrane domains in the inner/cytoplasmic leaflet of the plasma membrane is currently unknown.

### 1.2.2 Ceramide and ceramide enriched platforms

Sphingomyelin is predominantly present in the outer leaflet of the cell membrane. Sphingomyelin can be hydrolyzed to ceramide, which dramatically alters the biophysical properties of the plasma membrane. Ceramide constitutes the hydrophobic backbone of all complex sphingolipids and are composed of D-erythro-sphingosine and a fatty acid containing 2–36 carbon atoms in the acyl chain (Sandhoff, 2010). The amino alcohol binds to fatty acid forming an amide ester. Generally, fatty acyl chains are saturated or monounsaturated and an OH group might link to C2 or to the terminal carbon atom. Among all, the most abundant amount of physiological ceramides are those with the long (C16–20) and very long (C22–24) acyl chains (Fahy et al., 2005; Sandhoff, 2010). Therefore, structure of ceramides determines their poor solubility in water and cannot exist in biological fluids or in the cytosol (Goni et al., 2005). Ceramide molecules have the tendency to spontaneously self-associate. With the hydroxyl functional group, the amide linkage and the OH group on the sphingosine backbone, ceramides form extensive hydrogen bonds, which contribute to their high bulk transition temperature and in-plane phase separation, finally promote the formation of ceramide-enriched domains, particularly solid-like condensed domains in bilayers and monolayers (Castro et al., 2014; Maula et al., 2011). Moreover, ceramide dramatically alters membrane properties with other lipids. Low concentration as 5 mol% ceramide is sufficient to spontaneously induce ceramide-enriched membrane domains formation in model membranes (Veiga et al., 1999). A large number of biophysical techniques have been used to detect complex phase behavior of lipid mixtures with ceramide, including nuclear magnetic resonance (Hsueh et al., 2002), atomic force microscopy (Chiantia et al., 2006), X-ray diffraction (Boulgaropoulos et al., 2012), fourier transform infrared spectroscopy (Boulgaropoulos et al., 2011), fluorescence spectroscopy (Castro et al., 2007; Silva et al., 2007) and fluorescence microscopy (Staneva et al., 2009).

Ceramides are generated from diverse pathways. Several enzymes located in specific subcellular compartments are involved in ceramide metabolism, and certain activation of molecule signaling can occure due to their sites of function. Various stimuli can activate different metabolic pathways resulting the formation of ceramides (Fig. 1.2.2 A)

(Ruvolo, 2003; Stancevic and Kolesnick, 2010). *De novo* pathway of ceramide synthesis is located in endoplasmic reticulum (Bartke and Hannun, 2009; Carpinteiro et al., 2008). Serine palmitoyltransferase, a rate limiting step of *de novo* pathway, condensates the serine and palmitate to form 3-keto-dihydroshingosine. 3-keto-dihydroshingosine is then reduced to dihydrosphingosine, a metabolite further acylated to dihydroceramide by ceramide synthase. Six isoforms of ceramide synthase are found in mammals and are responsible for synthesizing (dihydro)ceramides with distinct chain lengths (Pewzner-Jung et al., 2006). Finally ceramide is synthesized from dihydroceramide by dihydroceramide desaturase. Ceramide can be delivered to the Golgi by vesicular trafficking or the ceramide transfer protein CERT for further structure modification to other sphingolipids (Hanada et al., 2007; Yamaoka et al., 2004).

Sphingomyelinases (SMase) catalyze the breakdown of phosphodiester bond of sphingomyelin to ceramide and phosphorylcholine (Airola and Hannun, 2013; Gulbins et al., 2004). According to basis of optimal pH values required for their activation, sphingomyelinases can be distinguished to acid SMase (ASM), neutral SMase (NSM) and alkaline SMase (alk-SM) (Hannun and Obeid, 2008; Stancevic and Kolesnick, 2010). It has been several years that only acid and neutral SMase are considered involved in cell signaling, however recent studies have summarized potential signaling roles for alkaline SMases (Duan, 2006). Acid SMase is encoded by SMPD1 gene which gives rise to lysosomal and secretory ASM (Marathe et al., 1998). Neutreal SMase are encoded by SMPD2–5 which are translated into NSM1, NSM2, NSM3, and mitochondria-associated SMase (MA-SMase) (Hofmann et al., 2000). Alk-SMase is encoded by the ENPP7 gene (Duan et al., 2003). Various stimulations can activate the SMase pathway of ceramide generation including cytokines, viral and bacterial infections, death receptor ligands, differentiation agents, and anti-cancer drugs (Carpinteiro et al., 2008; Tchikov et al., 2011; van Blitterswijk et al., 2003), resuting the ceramide enriched domain formation in the plasma membranes.

The salvage pathway or sphingolipid recycling is also an important pathway for ceramide generation (Gillard et al., 1998; Kitatani et al., 2008). Ceramide generation from the catabolism of complex sphingolipids is finally broken down into sphingosine,

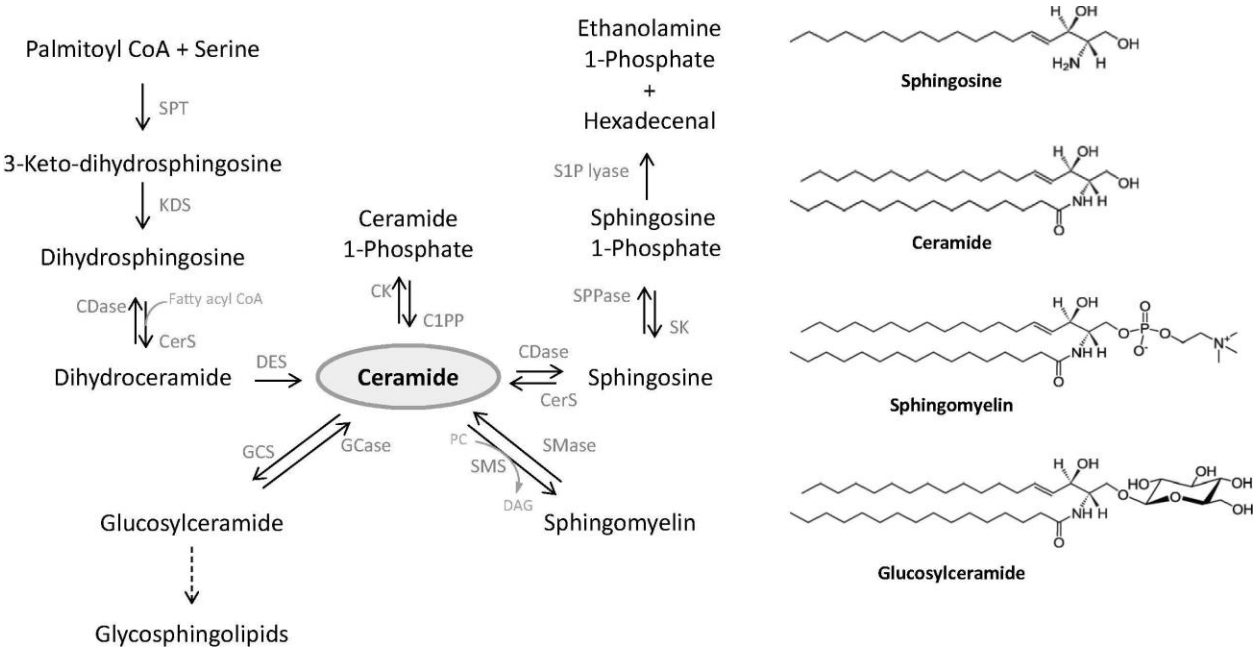



Figure 1.2.2 A. Ceramide structure and sphingolipid metabolism. Schematic representation of sphingolipid metabolism showing ceramide (Cer) as the central lipid. Ceramide is synthesized by *de novo* pathway, hydrolysis of sphingomyelin, salvage pathway and hydrolysis complex glycosphingolipids. The structures of sphingosine, C16-ceramide, C16-sphingomyelin and C16-glucosylceramide are shown in right panel. Abbeaviations: C1PP, ceramide 1-phosphate phosphatase; CDase, ceramidase; CerS, (dihydro)ceramide synthase; CK, ceramide kinase; DES, ceramide desaturase; GCase, glucosylceramidase; GCS, glucosylceramide synthase; KDS, ketosphinganine reductase; S1P lyase, sphingosine 1-phosphate lyase; SK, sphingosine kinase; SMase, sphingomyelinase; SMS, sphingomyelin synthase; SPPase, sphingosine phosphate phosphatase; SPT, serine palmitoyl transferase. (Castro et al., 2014)

which is then re-synthesized to ceramide through re-acylation. Several enzymes including SMases, glucocerebrosidase, ceramidases and ceramide synthases are involved in this pathway. In addition, several specific hydrolases break down complex sphingolipids leading to the generation of glucosylceramide and galactosylceramide, which is hydrolyzed into ceramide by specific β-glucosidases and galactosidases (Bartke and Hannun, 2009; Hannun and Obeid, 2008).

In general, the accumulation of ceramide within cellular membranes leads to the formation of ceramide-enriched membrane domains, which appears to sort proteins and to provide platform for the spatial recruiting receptors and intracellular signalling molecules upon various stimuli (Fig. 1.2.2 B) (Bollinger et al., 2005; Grassme et al.,

2007; Zhang et al., 2009). Ceramide plays important roles in a series of physiological and pathophysiological processes. First, the tightly packed ceramides strongly stabilize lipid rafts (Kolesnick et al., 2000; Xu et al., 2001). This stabilization of lipid rafts promote the formation of cell membrane domains into a lipid ordered state as mentioned above. Further, ceramide-enriched membrane domains serve the reorganization and clustering of receptor molecules, including CD95 (Grassme et al., 2001a; Grassme et al., 2001b), CD40 (Grassme et al., 2002), CD20 (Bezombes et al., 2004), FcγRII (Abdel Shakor et al., 2004), and CD28 (Boucher et al., 1995). Clustering of these receptors by ceramide may lead the very high receptor density, thus activating the downstream signaling molecules of the receptors, the exclusion of inhibitory molecules, conformational change

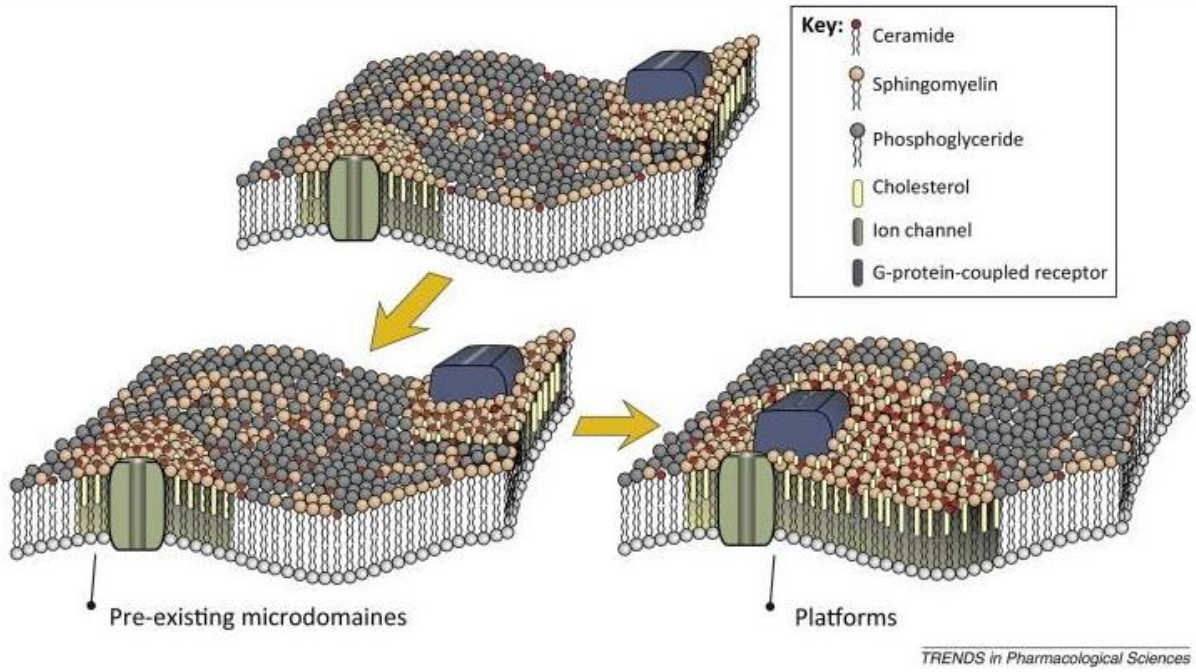



Figure 1.2.2 B. Ceramide action in biological membranes.

Biological membranes consist of sphingolipids (mainly sphingomyelin), cholesterol, and phosphoglycerides. Sphingolipids and cholesterol spontaneously interact with each other and separate from other phospholipids into distinct microdomains named rafts. The accumulation of ceramide, which is present ten times less abundant than sphingomyelin under normal conditions, changes the biophysical properties of these domains in the biological membranes. Ceramide molecules tend to self-associate and form ceramide-enriched microdomains, which can further fuse to large ceramide-enriched platforms. These ceramide-enriched membrane platforms function in recruiting and clustering of receptor molecules (Kornhuber et al., 2014).

of the receptor, and stabilization of the interaction of the receptor with its ligand (Grassme et al., 2007). In addition, ceramide also function as second messenger. Ceramide has been shown interacting and activating different enzymes such as cathepsin D (Heinrich et al., 1999; Zebrakovska et al., 2011), phospholipase A2 (Bharath et al., 2015; Huwiler et al., 2001), kinase suppressor of Ras (Zhang et al., 1997), ceramide-activated protein serine–threonine phosphatases (Dobrowsky and Hannun, 1993), and protein kinase C isoforms (Hage-Sleiman et al., 2016). Recent studies have identified an important function of ceramide in infectious biology. Basically, ceramides seem to be involved in the interaction of pathogens with host receptors, receptor clustering, and intracellular signaling molecules. Several studies have shown the ceramide interaction of pathogens with host, *Pseudomonas aeruginosa* (Becker et al., 2010; Pewzner-Jung et al., 2014), *Staphylococcus aureus* (Peng et al., 2015), *Neisseriae gonorrhoeae* (Grassme et al., 1997), and viruses (Aktepe et al., 2015; Dai et al., 2015; Mueller et al., 2014). In summary, ceramide and ceramide-enriched membrane domains might act as a crucial motif to reorganize the topology of a given signalosome, thus permit the stress stimulation and receptors to transmit biophysiological signals into the cell.

### 1.2.3 Acid sphingomyelinase

Acid sphingomyelinase (ASM), a hydrolase first identified by Gatt and colleagues in 1963 (Gatt, 1963), plays an important role in sphingolipids metabolism breaking down the sphingomyelin to ceramide and phosphorycholine. Acid sphingomyelinase (EC 3.1.4.12; gene symbol SMPD1 for human and Smpd1 for murine) is 5-6 kb long and localizes to chromosome 11p15.1–11p15.4 containing six exons and five introns (da Veiga Pereira et al., 1991; Schuchman et al., 1992), which is cloned and sequenced from human placenta (Quintern et al., 1989). Human ASM cDNA codes a polypeptide of 629 amino acids (Quintern et al., 1989; Schuchman et al., 1991) which shares approximately 82% amino acid identities to mouse acid sphingomyelinase (Newrzella and Stoffel, 1992). ASM is composed of three main domains: the N-terminal saposin domain, the proline-rich connector, and the catalytic domain (Gorelik et al., 2016; Xiong

et al., 2016). Deficiency of ASM results in the accumulation of sphingomyelin and causes lysosomal storage diseases, which are fatal neuropathic and visceral disease Niemann-Pick type A and a visceral anomalies disease Niemann-Pick type B (Brady et al., 1966; Schuchman, 2007; Schuchman et al., 1992).

Originally studies have reported that ASM effects solely lysosomal at an optima pH of 4.5-5.0 (Fowler, 1969), however recent studies suggest ASM catalyze the hydrolysis of LDL-sphingomyelin at a higher even neutral pH on the plasma membrane (Schissel et al., 1998a; Schissel et al., 1998b). The possible mechanism for the activity of different pH is that one single ASM gene generates two distinct enzymes: a lysosomal form ASM (L-ASM) and a secretory form ASM (S-ASM). The generation of two forms of ASM results from alternative modification and trafficking. First, the mutation of N-glycosylation sites affects the L-ASM and S-ASM catalytic activity and intracellular process (Ferlinz et al., 1997). The lysosomal trafficking of ASM is regulated by mannose-6-phosphorylation (M6P) receptor system in studies using fibroblasts with I-cell disease (Ferlinz et al., 1997; Takahashi et al., 2005). Other mechanisms have been reported that a trans-Golgi network (TGN) transmembrane protein sortilin is suggested to play a critical role in L-ASM trafficking along a Golgi-dependent route (Ni and Morales, 2006; Vazquez et al., 2016; Wahe et al., 2010). The pre-pro-form of ASM with a 75kDa (65 kDa protein core) molecular weight enter in the Golgi thereby generating the pro-form of ASM with a 72-75 kDa (63-64 kDa protein core) (Kornhuber et al., 2015). The S-ASM is released onto the outer leaflet of plasma membrane of a 75-80 kDa (64kDa protein core) protein molecular weight via the Golgi secretory pathway (Jenkins et al., 2010; Jenkins et al., 2011), whereas the L-ASM matures and traffics to lysosome as a 57 kDa (43 kDa protein core) enzyme (Edelmann et al., 2011) or a 65 kDa (55 kDa protein core) enzyme (Jenkins et al., 2011). Further, activation of S-ASM is dependent of exogenous $Zn^{2+}$ while L-ASM binds to $Zn^{2+}$ ions on its way to lysosomal compartments resulting the independence of exogenous $Zn^{2+}$ (Schissel et al., 1998b). Although several groups have studied the ASM, only a few of them discuss about the precise molecular mechanism in the regulation of lysosomal form of ASM and secretory form of ASM. It is

still unclear whether L-ASM and S-ASM hydrolyze differently from sphingomyelin pools, how it would cause different signaling in the cell.

Various stimulations can regulate the activation of ASM. The enzyme can be directly activated by oxidation (Zhang and Li, 2010). Studies have shown oxidation of purified ASM by hydrogen peroxide at C-terminal residue (Cys629) resulting in enzyme dimerization and activation (Qiu et al., 2003). Activation of ASM by DR5 or $Cu^{2+}$ is inhibited by reactive oxygen radical scavengers (Dumitru et al., 2007; Lang et al., 2007), although these studies do not conclude whether ASM activity is directly modulated by reactive oxygen species or by unknown intermediates. In addition, protease involves in the ASM activation. Inhibition of caspases by Ac-YVAD-chloromethylketone blocks the activation of ASM by CD95 (Brenner et al., 1998). Studies have demonstrated TNF-α initiates the interaction of the TNF-receptor with caspase-7, which mediates the proteolytic cleavage as a mode of ASM activation (Edelmann et al., 2011). Furthermore, isoforms of preotein kinase C (PKC) has been also shown to mediate the ASM activation. PKC δ serves as a key upstream kinase mediated phosphorylation of ASM at serine 508 (Zeidan and Hannun, 2007), which is recently confirmed by PKC δ-specific inhibitor abolishs the activation of ASM (Tsukamoto et al., 2012). Moreover, receptors such as CD95 (Dumitru and Gulbins, 2006; Grassme et al., 2001a; Grassme et al., 2001b) or DR5 (Carpinteiro et al., 2008), infection with bacteria (Grassme et al., 2003) or viruses (Avota et al., 2011; Grassme et al., 2005; Shivanna et al., 2015), induce the translocation and activation of ASM onto extracellular leaflet. Upon CD95 stimulation, vesicles containing ASM traffic to plasma membrane which is mediated by SNARE protein syntaxin 4, thus ASM is exposed to outer leaflet of plasma membrane (Perrotta et al., 2010). Finally, ASM is activated upon stress stimuli such as, UVA light (Charruyer et al., 2007; Zeidan et al., 2008b), radiations and chemotherapeutic drugs (Garcia-Barros et al., 2003; Lovat et al., 2004; Perrotta et al., 2007). Overall, although molecular mechanisms regarding regulation of acid sphingomyelinase are still partially known, the above studies elucidate several possible mechanisms of ASM activation.

Among these above mechanisms of ASM regulation, the interaction of ASM with reactive oxygen species (ROS) is one of the best studied. Our group has recently

shown hydrogen peroxide induces the activation and translocation of ASM in different cells (Li et al., 2012; Manago et al., 2015; Zhang et al., 2008). On the other hand, studies suggest the inhibition of ROS by several ROS scavenger TIRON, N-acetylcysteine (NAC), superoxide dismutase (SOD), catalase blocks the activation of ASM induced by different stimulation (Dumitru and Gulbins, 2006; Grammatikos et al., 2007; Lang et al., 2007; Zhang et al., 2008). Mechanically, the superoxide production induced activation of ASM is inhibited by a nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase inhibitor diphenyleneiodonium chloride (DPI) (Zhang et al., 2008). Genetic silence of a NADPH oxidase subunit $gp91^{phox}$ inhibits lipid raft formation induced by ASM activation (Boini et al., 2010). Another possible mechanism is that the direct oxidation of ASM at C-terminal cysteine residue 629 results in the activation of enzyme (Qiu et al., 2003). These evidences indicate ROS is required for the activation of ASM, although it is still unclear whether ROS regulates the enzyme activity directly or indirectly with other unknown mechanisms.

### 1.2.4 ASM and ceramide system in bacterial infection

Several reviews have shown ASM-ceramide system plays critical role in a wide range of cellular bioprocesses, such as cell death, proliferation, growth and differentiation (Beckmann et al., 2014; Henry et al., 2013; Perrotta et al., 2015; Wasserstein and Schuchman, 1993; Zeidan and Hannun, 2010). The alteration of ASM-ceramide system is involved in several pathological processes, such as genetic diseases (Aykut et al., 2013; Ranganath et al., 2016), tumor development (Carpinteiro et al., 2015; Carpinteiro et al., 2016), brain function and behavior (Grassme et al., 2015; Gulbins et al., 2016a; Gulbins et al., 2016b; Gulbins et al., 2013), atherosclerosis (Deevska et al., 2012; Kobayashi et al., 2013), and pathogenic infections (Avota et al., 2011; Gassert et al., 2009; Grassme et al., 1997; Grassme et al., 2005; Peng et al., 2015). Particularly, the interaction of bacterial infection and ASM-ceramide emerges as a novel research direction. Since ASM is located intracellular in lysosome and extracellular on the plasma membrane, and ceramide contribute to the formation of the plasma membrane, it is reasonable to expect ASM-ceramide system to be associated in the bioprocess of

bacteria invasion and killing. The studies related to ASM-ceramide sysyem and bacteria as well as bacteria generated toxin are listed in table 1.

| Bacteria/toxin | Mechanism | References |
|---|---|---|
| *P. aeruginosa* | IL-1 released, septic death | (Grassme et al., 2003) |
| | NADPH oxidase activation, ROS production | (Zhang et al., 2008) |
| | gp91phox clustering, ROS production | (Zhang et al., 2010) |
| | IL-8 release | (Yu et al., 2009) |
| | Pulmonary inflammation, cells death | (Teichgraber et al., 2008) |
| | CD95 clustering, epithelial cell death | (Becker et al., 2012) |
| *S. aureus* | Cytochrome C release, cell apoptosis | (Esen et al., 2001) |
| | ROS formation, tight junction degradation | (Peng et al., 2015) |
| *M. avium* | Granuloma formation | (Utermohlen et al., 2008) |
| *M. marinum* | Macrophage necrosis | (Roca and Ramakrishnan, 2013) |
| *M. tuberculosis* | Phagosome maturation | (Vazquez et al., 2016) |
| *L. monocytogenes* | Fusion of late phagosomes with lysosomes | (Schramm et al., 2008) |
| | Cytokine release, reactive nitrogen release | (Utermohlen et al., 2003) |
| *N. gonorrhoeae* | CEACAM receptor-mediated phagocytosis | (Hauck et al., 2000) |
| | Activation of PC-PLC | (Grassme et al., 1997) |
| *N. meningitidis* | Internalization of bacteria | (Simonis et al., 2014) |
| *E. coli* | Dendritic cells apoptosis | (Falcone et al., 2004) |
| | Cytokine release | (Hedlund et al., 1998) |
| *Salmonella* | ROS generation | (McCollister et al., 2007) |
| *P.acnes* | Hijacking host ASM | (Nakatsuji et al., 2011) |
| LPS | Dendritic cells apoptosis | (Falcone et al., 2004) |
| | TNF-α and MIP-2 release | (Jozefowski et al., 2010) |
| | Pulmonary inflammation | (McCollister et al., 2007) |
| | Endothelial cell apoptosis | (Haimovitz-Friedman et al., 1997) |
| Pyocyanin | Neutrophil cell death, ROS release | (Manago et al., 2015) |
| α-toxin | α-toxin binding, host cell necrosis | (Brauweiler et al., 2013) |

Table 1 Bacterial infection and acid sphingomyelinase/ceramide system.

It was firstly shown in 2001 that *S. aureus* infection triggers ASM activation and ceramide production in human endothelial cells (Esen et al., 2001). Genetic deficiency of ASM significantly inhibits the cell death of human fibroblasts triggered by *S. aureus*, which regulates the stimulation of JNK signaling pathway and alteration of mitochondrial function. The functional inhibition of JNK by Tam67 gene transfection prevents the *S. aureus* induced cell apoptosis. These results are consistent with the finding that ASM and ceramide enriched platforms mediate macrophage apoptosis via stimulation of JNK upon *P. aeruginosa* infection (Zhang et al., 2008).

Recently, it was found that genetic deficiency or pharmacological inhibition of ASM protects mice against pneumonia and lethal *S. aureus* sepsis (Peng et al., 2015). ASM is activated by *S. aureus* in endothelial cells thereby the ceramide enriched platforms are generated. ASM activation triggers the release of superoxide whereas the ASM is inhibited by antioxidants. The ASM-ceramide system and ROS acts as a positive feedback loop mechanism upon *S. aureus* infection which is similar to the findings of previous studies (Zhang et al., 2008). ASM-ceramide triggered superoxide production induces degradation of tight junction proteins ZO1, ZO2, occluding and E-cadherin upon *S. aureus* infection *in vitro* or *in vivo*, which was reduced by inhibition of ASM by amitriptyline or antioxidants. Several evidences have demonstrated that the superoxide is responsible for the degradation of tight junctions via proteolytic enzymes matrix metalloproteinases (MMP) (Abdul-Muneer et al., 2015; Gu et al., 2011; Rochfort et al., 2014). Moreover, ASM has been shown positively regulating the transcription of MMP mRNA and expression of protein (Bauer et al., 2009a; Bauer et al., 2009b; Butler et al., 2007).

*S. aureus* is a primary cause of sepsis and lethal lung edema even with the treatments of antibiotics clinically. A series of antibiotics were used to clear the bacteria burden in the mice however the mice still died because of lung edema. Mice with ASM inhibitor amitriptyline treatments or ASM gene deficiency reduces the lung edema by reducing the degradation of tight junction and preventing the myeloid cell trafficking (Peng et al., 2015). However, the bactericidal capacity is also reduced since dysfunction of ASM results the fail of clustering and activating NADPH oxidase, resulting the susceptibility and high mortality of mice to the *S. aureus* infection. A combination of antibiotics with ASM deficiency or pharmacological inhibition successfully rescues the mice from lethality of *S. aureus* infection. This combination is sufficient to clear the bacteria with a protection of tight junction protein degradation which suggesting a novel therapeutic approach to treat lung edema and bacteremia in *S. aureus* induced sepsis.

Staphylococcal alpha-toxin (α-toxin) appears to function by forming pores in cell membranes, damaging the membrane permeability, eventually triggering the cell death. α-toxin is secreted as water soluble monomer protein, once they reach the targets they

are capable of binding and oligomerization into a heptameric structure, therefore inserting or translocating across the cell membrane of the host (Gouaux et al., 1994; Song et al., 1996). ASM-ceramide system is found protecting against staphylococcal α-toxin induced keratinocyte death (Brauweiler et al., 2013). Filaggrin is a protein critical for epidermal skin barrier function (Irvine et al., 2011). α-toxin targets and damages the filaggrin deficient or undifferentiated keratinocytes. The differentiation process leads to a significant increase of expression of ASM mRNA and protein. Gene silence of filaggrin reduces the both the amount and activity of secreted ASM but not the intracellular ASM activity. ASM is sufficient to protect cell death against α-toxin by reducing expression of α-toxin receptors and the binding of α-toxin with receptors (Brauweiler et al., 2013).

ASM-ceramide system is also involved in several other host bacteria infection. Upon *Pseudomonas aeruginosa* (*P. aeruginosa* ) infection, ASM is activated and translocated to the extracellular leaflet of the plasma membrane which stumilates the ceramide generation and platform formation, thus mediates the bacteria internalization and killing, cytokine such as IL-1β release, cell death, inflammatory response, and susceptibility to bacteria challenge (Grassme et al., 2003; Teichgraber et al., 2008; Yu et al., 2009; Zhang et al., 2008; Zhang et al., 2010). Further, ASM generated ceramide promotes bacteria killing and cell death in the infection of macrophages with pathogenic mycobacteria (Roca and Ramakrishnan, 2013; Vazquez et al., 2016). ASM deficiency highly impairs the bactericidal capacity of mice challenging the *Listeria Monocytogenes* (*L. Monocytogenes*), which due to the fail of macrophages intracellular bacteria killing involving the listeriocidal proteases cathepsin D, B and L (Utermohlen et al., 2003). In addition, ASM generated ceramide mediates interaction of pathogenic *Neisseria* with host receptors carcinoembryonic antigen related cellular adhesion molecule (CEACAM) or tyrosine kinase ErbB2 regulated phagocytosis in phagocytic or non-phagocytic cells (Grassme et al., 1997; Hauck et al., 2000; Simonis et al., 2014). The activation of ASM and generation of ceramide and their exposure to NO depend on the cGMP formation during the infection process of *Escherichia coli* (*E. coli*) (Falcone et al., 2004). Salmonella triggers a significant increase in the secreted fraction of ASM (McCollister et al., 2007). Recent studies have shown host ASM also involves in the *P. acnes* virulence

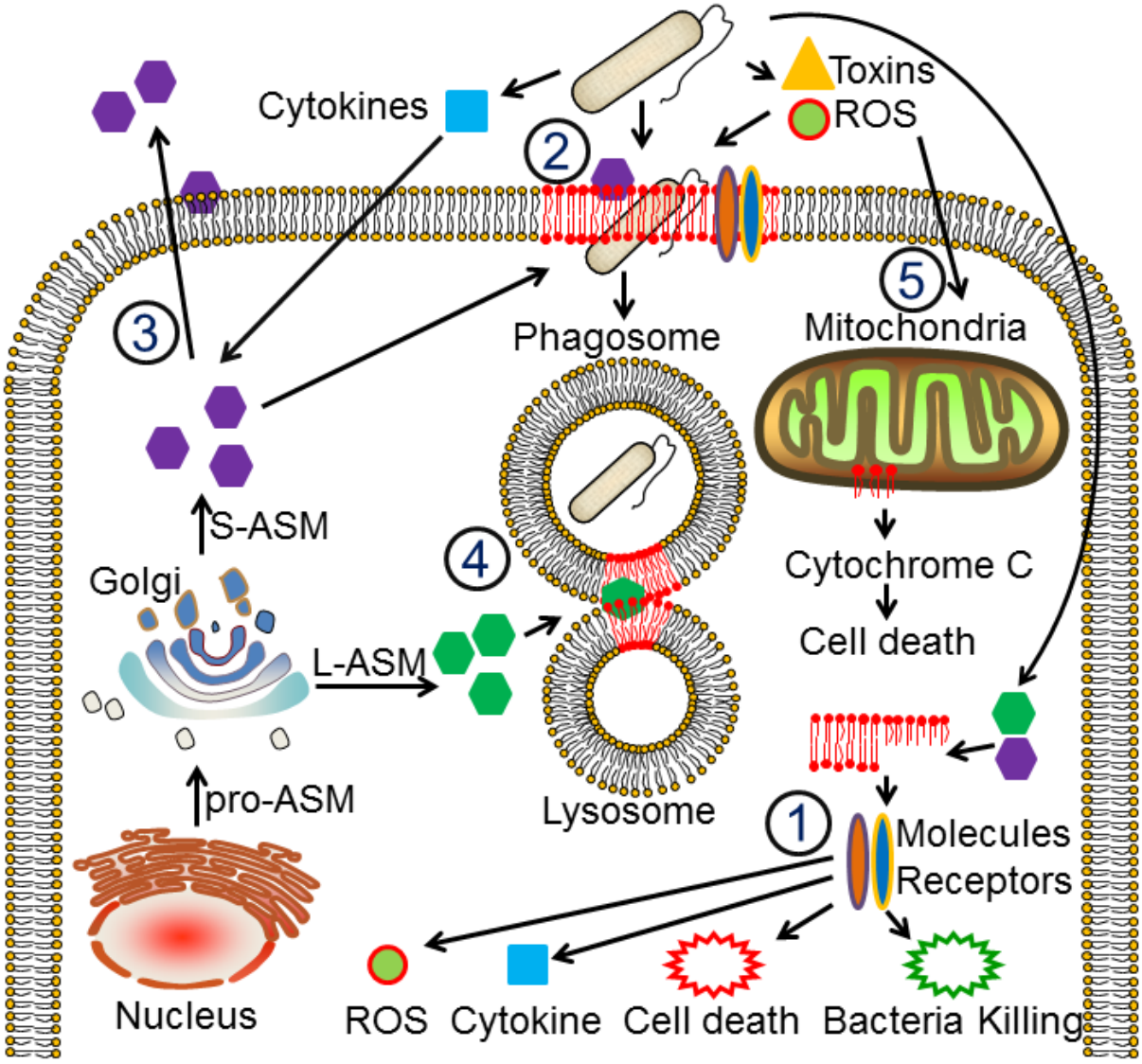



Figure 1.2.4 ASM-ceramide system in the bacteria-host interaction.

1. Overview of ASM in the bioprocess of infection. Bacteria induce activation of ASM and release of ceramide, which recruit signaling molecules and receptors, such as NADPH oxidase, ErbB2, JNK, CFTR, CD95, JNK, and p38 kinase, therefore modulating ROS generation, cytokine release, host cell death, and bacterial killing. 2. ASM generated ceramide recruit molecules and receptor and mediate bacteria internalization. 3. Inflammatory cytokines including TNF-α and IL-1β stimulates the secretion of ASM. 4. ASM-ceramide system is needed for phagolysosome maturation. 5. ASM-ceramide system is involved in mitochondria induced cell death upon infection.

induced inflammation (Nakatsuji et al., 2011).

ASM-ceramide system has been shown to be crucially in the regulation of host interaction with bacteria, including *P. aeruginosa*, *S. aureus*, mycobacteria, *L monocytogenes*, *Neisseria*, *E. coli*, Salmonella, *P. acnes* and bacteria toxins. Fig. 1.2.4 shows the presumable role of ASM-ceramide system in the bacterial infection process on the cells. The infection of mammalian cells with bacteria triggers the activation of ASM and secretion of ASM onto membrane and extracellular environment. The ASM generated ceramide initiate the lipid raft organization thereby mediating the bacteria acting on host cells. ASM facilitates the activation of NADPH oxidases which involves generating of superoxide, known to be responsible for bacteria killing and regulate the cell apoptosis. The ceramide platforms critically mediate the internalization of bacteria into host cells. Moreover, ASM generated ceramide modifies the membrane biophysical properties and recruits receptor molecules, thereby modulating the fusion of phagosome and lysosome. In addition, ASM have an influence on the cytokine release, inflammatory response, and the susceptibility of mice. Taken together, although the detail mechanism of ASM-ceramide system acting on bacterial infection remains to be further studied, strong evidences shows the central role of this system in bacteria host interactions. Targeting the ASM-ceramide system is a novel potential therapeutic approach for treating bacterial infection.

## 1.3 CD44

### 1.3.1 Structure of CD44

CD44 is a widely expressed glycoprotein on the surface of a diverse variety of cell types, including endothelial cells, epithelial cells, chondrocytes, fibroblasts, keratinocytes, neural cells and leukocytes (Orian-Rousseau and Ponta, 2015; Sherman et al., 1994). CD44 is a transmembrane hyaluronan-binding protein which mediates the cell adhesion and migration in multiple physiological and pathophysiological processes, such as tumor development and metastasis (Karousou et al., 2016; Misra et al., 2015), wound healing (Prosdocimi and Bevilacqua, 2012; Tolg et al., 2014), inflammatory diseases (Johnson and Ruffell, 2009), leukocyte extravasation (McDonald and Kubes, 2015), neurological disorders (Dzwonek and Wilczynski, 2015), bacterial and viral infections (Abe et al., 2012; Garay et al., 2016).

The CD44 proteins range in molecular weight from 80 to 250 kDa and encoded by a single highly conserved gene (Screaton et al., 1992). The variation of CD44 protein forms is partially resulted from post-translational modifications, which depends on the cell type and growing status. Moreover, alternative splicing, the regulated alternative usage of exons during pre-mRNA splicing, affects the CD44 transcription mainly of extracellular, membrane-proximal stem structure of CD44 proteins (Gunthert et al., 1991; Stamenkovic et al., 1991).

Structurally, the protein consists of three regions, an amino-terminal domain, a stem structure, and a transmembrane and cytoplasmic-tail region (Fig. 1.3.1). The hyaluronan-binding amino-terminal globular protein domain is encoded by non-variable exons 1 to 5 of CD44, which is considerable conserved between mammalians and is recognized to fold into a globular tertiary structure by the forming disulphide bonds between three pairs of cysteine residues (Goodison et al., 1999). This domain serves as a docking site for multiple components of the extracellular matrix (ECM) and interacts with ECM such as hyaluronan (Evanko et al., 2015; Konopka et al., 2016), fibronectin (Pal et al., 2013; Viana et al., 2015), collagen and laminin (Damodarasamy et al., 2014; Garrett et al., 2007; Golan et al., 2016), although the binding mechanism has not been

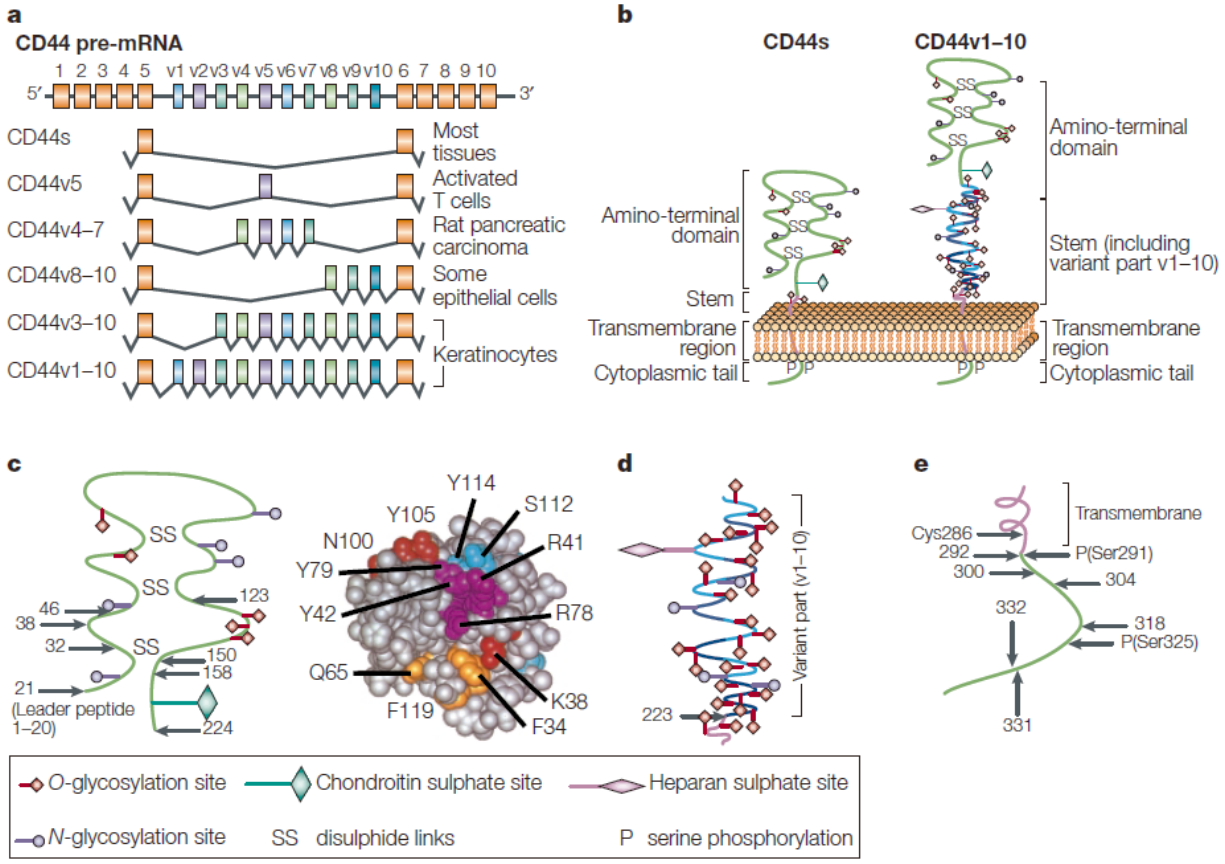



Figure 1.3.1 CD44 transcripts and proteins.

a. 20 exons encode the CD44 pre-mRNA, 10 of which are variant exons regulated by alternative splicing. b. The smallest CD44 isoform (CD44s) and largest variant isoforms CD44v1–10 shows that the sequences encoded by the variant exons are in the stem region. c. The amino-terminal domain of CD44 proteins contains the hyaluronan-binding motifs and a basic motif outside the link domain. The interchain disulphide formed by cysteine residues is important for the stability of the link module. d. The stem structure of CD44s is composed of 46 or up to 381 variant-exon-encoded amino acids in human or 423 in mouse amino acids, as shown for CD44v1–10. e. The cytoplasmic domain supports the binding of proteins which is critical for cytoskeletal organization and signalling. Amino acids 292–300 are binding sites for Ezrin, radixin, moesin (ERM) proteins and amino acids 304–318 are for ankyrin. Basolateral distribution of CD44 is related to amino acids 331 and 332 (Ponta et al., 2003).

studied precisely. Further, the domain, containing 90 amino acid residues from 32 to 123, is considered as a link domain which enables the binding of CD44 with hyaluronan and other glycosaminoglycans (Sherman et al., 1994; Sleeman et al., 1997). This hyaluronan binding motif presents similar homology with both the cartilage link protein and with the proteoglycan core protein (Naor et al., 1997).

The stem structure of CD44 contains 46 amino acids and separates the amino-terminal globular domain of the smallest CD44 isoform (the standard isoform or CD44s) from cell membrane. This stem contains putative proteolytic cleavage sites for metalloproteinases ADAM-10 and ADAM-17, or membrane type 1-matrix metalloproteinase (Nagano and Saya, 2004; Okamoto et al., 1999). The stem structure can be enlarged to 381 amino acids in human by sequences which are encoded by alternatively spliced variant (v) exons of CD44. The inclusion of variant exons is partially dependent on mitogenic signals which modulate alternative splicing (Konig et al., 1998; Weg-Remers et al., 2001). For example, sequence encoded by exon v3 contains a heparan-sulphate site interacting with heparin-binding protein (Suga et al., 2012).

The highly conserved cytoplasmic region is encoded by part of C-terminal exon 18, 19 and 20 (Goldstein and Butcher, 1990). The hydrophobic transmembrane domain is encoded by conserved exon 18 and is composed of 23 hydrophobic amino acids and a cysteine residue. Importantly, the transmembrane region is critically involved in lipid raft-mediated regulation of Hyaluronan-CD44 Interactions (Murai, 2015; Neame et al., 1995). Binding of intracellular proteins with cytoplasmic tail regulates the interaction of CD44 with the cytoskeleton. The first identified CD44 binding protein ankyrin mediates contact with the cytoskeletal component spectrin (Lokeshwar and Bourguignon, 1991; Wang et al., 2014). In addition, ezrin, radixin and moesin proteins (ERM), importantly for regulation of cell shape and migration, interact with a basic-amino-acid motif in the cytoplasmic tail of CD44. Recent studies have implicated Smad1 interactions with activated CD44 bound to ERM protein and linked to actin cytoskeletons (Mori et al., 2008). Phosphorylation of Ser291 modulates the interaction between CD44 and ezrin *in vivo* which is critical for CD44-dependent directional cell motility (Legg et al., 2002). Cytoplasmic tail of CD44 also regulates the activation of Rho family of GTPase (Bourguignon, 2008; Bourguignon et al., 2010; Ohata et al., 2012).

### 1.3.2 Mechanism of CD44 function

CD44 independently or in collaboration with other cell surface or intracellular molecules induces multiple biological activities, including inflammatory response, cell death and proliferation, and cytoskeleton reorganization. The signaling output of CD44-involved pathway is shown in Fig. 1.3.2 (Naor et al., 2002). Presumably, function of CD44 can be proposed as three parts: First, CD44 serves as a ligand-binding receptor by interacting with ECM or presents as a specialized 'platform' for growth factors and matrix metalloproteinases (MMPs). Second, CD44 can function as a co-receptor to activate growth factor receptors. Third, CD44 is involved in reorganization of cortical actin cytoskeleton (Ponta et al., 2003).

CD44 functions as a ligand-binding surface protein and interact with soluble extracellular components as well as ECM. These interactions trigger cellular responses and a passive adhesive function, or represented as a cell-surface protein that binds enzymes and their substrates. The binding affinity of CD44 with hyaluronan is regulated intracellular demonstrated by mitogenic stimulated upregulation of binding affinity and by phosphorylation of serine residues in the cytoplasmic tail of CD44 (Orian-Rousseau and Ponta, 2015; Vigetti et al., 2014). Regulation of binding affinity is critical for leukoctye migration and rolling. Studies have shown proteolytic cleavage might be another mechanism of regulated CD44–hyaluronan binding (Okamoto et al., 1999; Okamoto et al., 2002). The inhibition of CD44 cleavage leads to the block of tumor cell migration on a hyaluronan substrate. In addition, CD44 is implicated in several passive functions which possibly do not require direct activation of signalling cascades. For instance, hyaluronan can function as an adhesive bridging molecule between cells. CD44 is also involved in axon pathfinding.

CD44 proteins can function in trapping and concentrating molecule relevant for growth, and bringing substrates and enzymes into interacting with one and another. CD44 recruits MMP9, an enzyme which facilitates degradation of collagen IV and regulates tumor cell invasion on the plasma membrane (Miletti-Gonzalez et al., 2012). Activating of precursor pro-form of transforming growth factor (TGF)-beta by MMP9 requires its

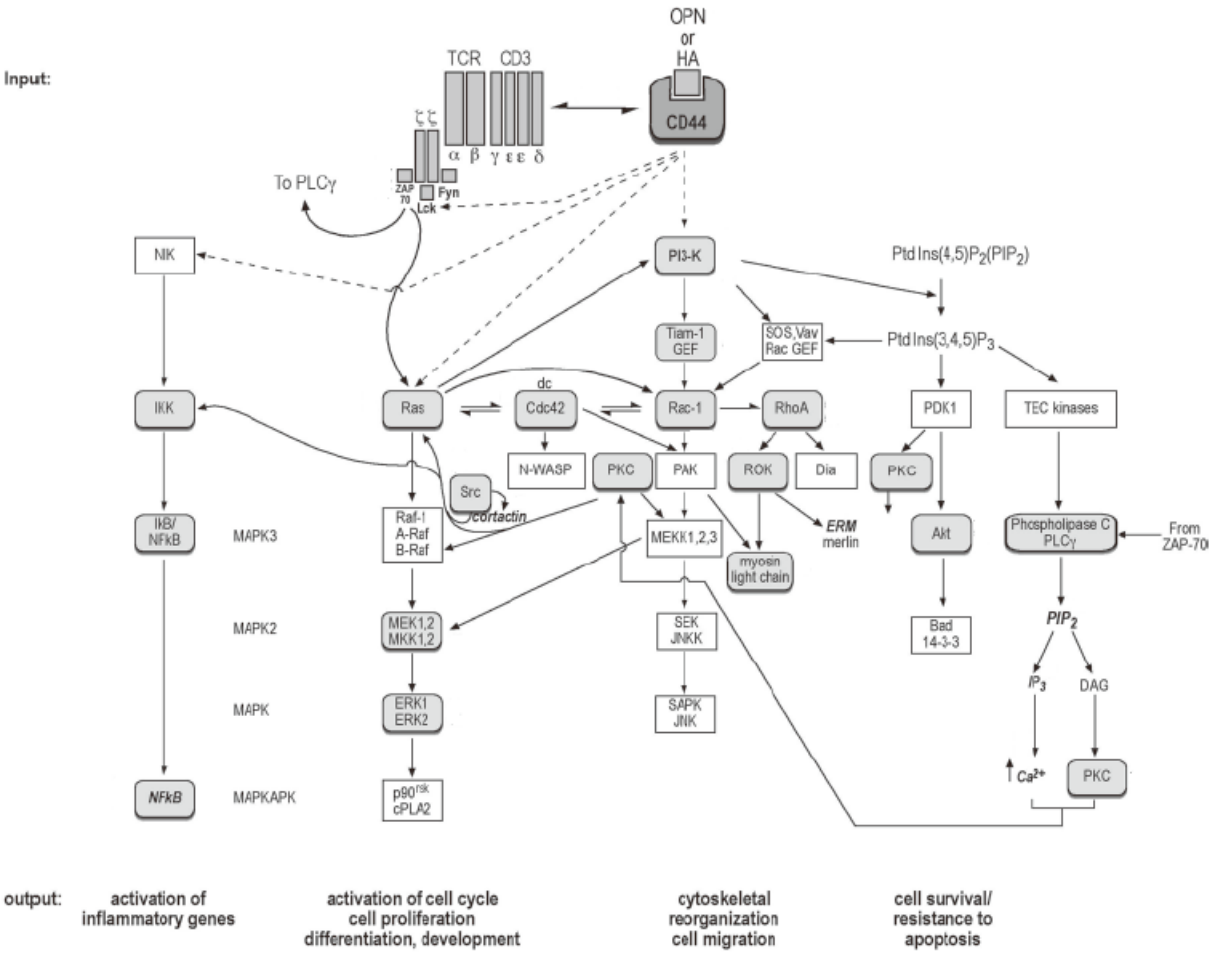



FIGURE 1.3.2 CD44-involved signal transduction pathway and function.

CD44 either independently or in collaboration with other signal transduction molecules (shadowed boxes and the bold, non-framed abbreviations) regulates multiple biological signaling pathways. The broken lines implicate the coupling mechanism between this receptor and the signaling elements need to be elucidated. CD44 connected TCR with double-headed arrow shows the structural and functional association between these two receptors. Abbreviations: Cdc42, homologous to yeast cell division cycle gene 42; ERK, extracellular signal regulated kinase; GEF, guanine exchange factor; HA, hyaluronic acid; IκB, inhibitor of NK-κB; IKK, IκB kinase; MAPK, mitogen-activated protein kinase; MAPKAPK, mitogen-activated protein kinase activated protein kinase; MEK, MAPK/ERK kinase; MEKK, MEK kinase; NIK, NF-κB-inducing kinase; OPN, osteopontin; PAK, p21-activated kinase; PI3-K, phosphatidylinositol 3-OH-kinase; PKC, protein kinase C; PLC, phospholipase C ;Ptd Ins 4,5 P2, phosphatidylinositol-4,5-biphosphate; Ptd Ins 3,4,5 P3, phosphatidylinositol-3,4,5-triphosphate; Rac, Ras-related C3-botulinum toxin substrate; Ras, rat sarcoma virus; Rho, Ras homologous; ROK, Rho-kinase; SAPK, stress-activated protein kinase; SEK, SAPK/ERK kinase; TCR, T cell receptor. (Naor et al., 2002)

binding to CD44 on the cell surface, thereafter the TGF-beta triggers neovascularization (Yu and Stamenkovic, 2000). Moreover, CD44 recruits MMP7 to the cell surface, a signaling pathway accounts for the suppression of apoptosis in tumor cells (Okayama et al., 2009). The isoforms of CD44 proteins containing exon v6- and v7-encoded sequences interact with phosphorylated acidic glycoprotein osteopontin (OPN) which has been shown in involving inflammation (Higashi et al., 2015; Liu et al., 2015). Deficiency in exons v6 and v7 results in significantly reduced inflammation on induction of experimental colitis (Denhardt et al., 2001).

CD44 can function as co-receptors in signal transduction. CD44 isoforms containing exon v6 sequences serve as a co-receptor for the activation of mesenchymal–epithelial transition factor (Met) (Ghatak et al., 2014; Taher et al., 1999). Activation of Met is dependent on CD44 variants with exon-v6-encoded sequences experession. CD44 also act as a co-receptor for the ErbB receptor tyrosine kinase family (Palyi-Krekk et al., 2008; Sherman et al., 2000). For example, v3 isoform of CD44 interferes with the CD44-EGFR/ErbB2 interaction, alters the ERK1/2 and p38 MAPK thereby regulating cell proliferation and migration (Hernandez et al., 2011). CD44 cytoplasmic tail interacts with multiple intracellular proteins which are function in signaling transduction. These proteins include Src, small Rho GTPase, Rho GDP-dissociation inhibitor (GDI), proto-oncogene protein-tyrosine kinases LCK and FYN and PKC (Naor et al., 1997). The platform and co-receptor functions together and are responsible for the action of CD44 in tumor development, inflammation and autoimmune disease.

CD44 interacting with proteins linked to actin cytoskeleton is crucial for actin dynamics. One of these proteins is ERM protein, which is required for the internalization of Met mediated by CD44 (Hasenauer et al., 2013). Met-mediated signaling from the endosomes depends on its collaboration with CD44v6 and the link to the cytoskeleton provided by ERM proteins. Cytoplasmic tail recruits ERM proteins to the complex of CD44v6, c-Met, and HGF, a process initiated by ERM interacting with coreceptor which is absolutely necessary for mediating the HGF-dependent activation of Ras (Orian-Rousseau et al., 2007). In addition, Hyaluronidase-2 (Hyal2), the major enzyme for hyaluronan metabolism, directly interacts with CD44 and inhibits the formation of

glycocalyx thereby suppressing ERM-related cytoskeletal interactions and diminishes cell motility (Duterme et al., 2009). These data implicate that the actin cytoskeleton linked ERM proteins plays an important role in the signal transduction regulated by CD44.

### 1.3.3 CD44 in bacterial infection

CD44 has been implicated in a variety of diseases, including cancer, arthritis, cardiovascular disease, wound healing and infections (Jordan et al., 2015; Orian-Rousseau and Ponta, 2015). Recent studies have demonstrated CD44 emerges as a new role in physiological and pathological processes of bacterial infection.

CD44 is crucially involved in host pathogen interaction, particularly in activation and migration of lymphocytes (Siegelman et al., 1999). CD44 has also been shown connected to generation of antimicrobial peptides (Hill et al., 2013). In addition, pathogens can utilize the CD44-HA signaling for progression of infections and resulting severe consequences. CD44 is required for the activation, homing, and extravasation of lymphocytes into inflammatory sites (DeGrendele et al., 1997; Denning et al., 1990). Hyaluronan derived from human milk induces generation of human β-defensin 2 (HβD2) which enhances resistance to infection in the intestinal epithelium (Hill et al., 2013). These studies have suggested that CD44–HA signaling stimulates protective antimicrobial defense during early infancy. Similarly, studies have implicated the contribution of CD44-HA to innate defense response which is TLR4-dependent (Hill et al., 2012).

Several studies have implicated CD44 directly or indirectly interacting with bacteria and host cells. For instance, capsular polysaccharide of group A *Streptococcus* (GAS) which has a similar size to mammalian cells adheres to human keratinocytes through its hyaluronan-rich polysaccharide capsule (Schrager et al., 1998). *In vivo* studies have shown transgenic mice with reduced CD44 expression significantly inhibits binding of CD44 with GAS and colonization (Cywes et al., 2000). In addition, molecular mass

difference of HA for macrophage-mediated phagocytosis of GAS can influence the GAS virulence. High molecular mass HA facilitates GAS deep tissue infections, whereas the generation of short-chain HA can be protective (Schommer et al., 2014).

CD44 is involved in pneumonia caused by *Escherichia coli*. Deficiency of CD44 results in increasing of several inflammation related mRNA expression and neutrophil accumulation (Wang et al., 2002). However, this is not observed in *Streptococcus pneumoniae*-induced pneumonia which may due to the latter expressing of hyaluronidase, in turn decreasing CD44–HA regulated signaling and the downstream induction of inflammation. Further studies have demonstrated CD44 facilitates bacterial outgrowth and dissemination during pneumococcal pneumonia, which in lethal infection results in a prolonged survival of CD44 KO mice (van der Windt et al., 2011). CD44 has been previously shown to play a critical role in resolving lung inflammation (Teder et al., 2002).

CD44 has been implicated interacting with infection process of several other bacteria including *Helicobacter pylori* (Khurana et al., 2013), *Listeria monocytogenes* (Jung et al., 2009), and *Shigella* (Lafont et al., 2002). CD44 deficiency or inhibition by a peptide PEP-1 generates significantly less proliferating isthmus stem cells than wild type after infection with *Helicobacter pylori*. CD44 is required for the entry of bacteria and localizes at the plasma membrane of cellular extensions induced by *Shigella* (Lafont et al., 2002). In addition, the collaboration of c-Met and CD44 contributes to the invasion of *L. monocytogenes* into host cells by the binding of a secreted bacterial protein IpaB. Once invaded intracellular, CD44 and ezrin become localized at the site of the membrane before protrusion formation (Sechi et al., 1997).

Taken together these studies implicate that CD44 signaling plays a central role in bacterial infection and host inflammatory response, which helps to develop various strategies for targeting of CD44 and CD44-based therapeutic interventions.

### 1.4 Aim of study

*Staphylococcus aureus* is one of the major human pathogen which causes a variety of clinical infections including pulmonary infections (Lowy, 1998; Tong et al., 2015). *S. aureus* has evolved multiple comprehensive mechanisms to avoid the killing by human immune system, thus to survive extreme conditions and replicate within the host (Liu, 2009). Antibiotics are preferred and effective treatment for *S. aureus* infections for a long time, however, antibiotic resistance becomes a severe threaten to this pathogen infection. Lack of efficacious ways treating staphylococcal infection leads to an increasing for clinical outcome even death and financial cost. It is important and urgent to find more therapeutic target for fighting against *S.aureus* infection.

Recent studies have implicated diverse functions of ceramide in infections, which suggest ASM-ceramide system plays an important role in the regulation of balance of the host and the microbe (Grassme and Becker, 2013). Our group has revealed that ASM-ceramide system regulates the interaction of several pathogens with host cell. In particular, our group firstly demonstrated in 2001 that *S. aureus* infection triggers ASM activation and ceramide production in human endothelial cells (Esen et al., 2001). Further, we identified that genetic deficiency or pharmacological inhibition of ASM protects mice against pneumonia and lethal *S. aureus* sepsis (Peng et al., 2015). In the study, ASM-ceramide triggered superoxide production induces degradation of tight junction protein in endothelial cells *in vitro* and *in vivo*. ASM deficiency or pharmacological inhibition prevents the myeloid cell trafficking. However, the mechanism of ASM-ceramide system interacting with macrophages in *S. aureus* induced lung injury even host death is still unknown. Macrophages are the most numerous immune-cells present in the lung environment which play a critical role in innate immunity and the clearance of *S. aureus* infection (Foster, 2005; Pozzi et al., 2015).

The present study was performed to investigate the role of the Asm-ceramide system in the infection of macrophages with *S. aureus*. We identified CD44 as a novel receptor for *S. aureus* binding. CD44 is intimately connected with the Asm, clusters in ceramide-enriched domains after infection, which amplifies CD44 signaling and results in further

activation of the Asm and thereby in a positive forward feedback loop between CD44 and the Asm. CD44 activation by *S. aureus* stimulates small-G proteins, a re-organization of the cytoskeleton, internalization of the pathogen and fusion of phagosomes with lysosomes, a process that requires again Asm. Deletion of CD44 or the Asm prevents internalization of *S. aureus*.

# 2. Materials

## 2.1 Chemicals

| | |
|---|---|
| Acetic acid | Merck KGaA, Darmstadt, Germany |
| Acetone | Merck KGaA, Darmstadt, Germany |
| Acrylamide ($C_3H_5NO$) | Carl-Roth GmbH & Co, Karlsruhe |
| Agarose | Gibco, Invitrogen,Karlsruhe, Germany |
| Ammonium persulfate (APS) | Carl-Roth GmbH & Co, Karlsruhe |
| Bovine serum albumin (BSA) | Sigma-Aldrich Chemie GmbH, Steinheim |
| β-mercaptoethanol | Sigma-Aldrich Chemie GmbH, Steinheim |
| Bromphenol blue | Sigma-Aldrich Chemie GmbH, Steinheim |
| Calcium chloride ($CaCl_2$) | Sigma-Aldrich Chemie GmbH, Steinheim |
| Cardiolipin | Sigma-Aldrich Chemie GmbH, Steinheim |
| Chloroform ($CHCl_3$) | Applichem GmbH, Darmstadt, Germany |
| CDP-STAR with Nitro-Block II enhancer | PerkinElmer, Boston, USA |
| Dithiothreitol | Carl-Roth GmbH & Co, Karlsruhe |
| Dimethylsulfoxid (DMSO) | Sigma-Aldrich Chemie GmbH, Steinheim |
| Enhanced chemiluminescence (ECL) | Thermo Scientific, USA |
| Ethanol ($C_2H_5OH$) | Sigma-Aldrich Chemie GmbH, Steinheim |
| Ethylenediamine Tetraacetic Acid | Serva Electrophoresis GmbH, Heidelberg |
| Fetal calf serum (FCS) | Gibco, Invitrogen, Karlsruhe |
| Formamide | Sigma-Aldrich Chemie GmbH, Steinheim |
| Gentamycin | Sigma-Aldrich Chemie GmbH, Steinheim |
| Glucose ($C_6H_{12}O_6$) | Sigma-Aldrich Chemie GmbH, Steinheim |
| Glycerol ($C_3H_8O_3$) | Fluka Chemie GmbH, Buchs |
| Glycine ($C_2H_5NO_2$) | Applichem, GmbH, Darmstadt, Germany |
| HEPES | Carl-Roth GmbH & Co, Karlsruhe |
| Hydrochloric acid (HCl) | Sigma-Aldrich Chemie GmbH, Steinheim |
| Isopropanol | Sigma-Aldrich Chemie GmbH, Steinheim |
| Ketamine | Ceva Tiergesundheit GmbH, Duesseldorf |
| Magnesium chloride ($MgCl_2$) | Sigma-Aldrich Chemie GmbH, Steinheim |

| | |
|---|---|
| Magnesium sulphate ($MgSO_4$) | Sigma-Aldrich Chemie GmbH, Steinheim |
| Methanol | Fluka Chemie GmbH, Buchs |
| Monopotassium phosphate ($KH_2PO4$) | Merck KGaA, Darmstadt, Germany |
| Mowiol | Kuraray Specialities GmbH, Frankfurt |
| NP-40 (Igepal) | Sigma-Aldrich Chemie GmbH, Steinheim |
| Paraformaldehyde (PFA) | Sigma-Aldrich Chemie GmbH, Steinheim |
| Pepsin | Invitrogen, Frederick, USA |
| Potassium chloride (KCl) | Sigma-Aldrich Chemie GmbH, Steinheim |
| Protease inhibitor | Carl-Roth GmbH & Co, Karlsruhe |
| Saponin | Serva Electrophoresis GmbH, Heidelberg |
| Sodium acetate ($CH_3COONa$) | Sigma-Aldrich Chemie GmbH, Steinheim |
| Sodium chloride (NaCl) | Carl-Roth GmbH & Co, Karlsruhe |
| Sodium dodecyl sulphate (SDS) | Serva Electrophoresis GmbH, Heidelberg |
| Sodium fluoride (NaF) | Sigma-Aldrich Chemie GmbH, Steinheim |
| Sodium hydroxide (NaOH) | Sigma-Aldrich Chemie GmbH, Steinheim |
| Sodium phosphate ($Na_2HPO_4$) | Merck, Darmstadt |
| Sodium pyrophosphate ($Na_4P_2O_7$) | Sigma-Aldrich Chemie GmbH, Steinheim |
| Tryptic soy broth (TSB) | BD Biosciences, Heidelberg, Germany |
| Tris-HCl and Tris-Base | Carl-Roth GmbH & Co, Karlsruhe |
| Triton X-100 | Sigma-Aldrich Chemie GmbH, Steinheim |
| Tween-20 | Sigma-Aldrich Chemie GmbH, Steinheim |
| Xylazin | Ceva Tiergesundheit GmbH, Duesseldorf |
| Xylene | Applichem GmbH, Darmstadt, Germany |

### 2.2 Antibodies

| | |
|---|---|
| Goat anti-Armenian hamster immunoglobulin G | Jackson ImmunoResearch, USA |
| Rabbit anti-*S. aureus* antibody IgG | Abcam, USA |
| Mouse anti-*S. aureus* antibody IgG | Abcam, USA |
| Rabbit anti-phospho-ERM antibody IgG | Cell Signaling Technology, USA |

| | |
|---|---|
| Rabbit anti-Ezrin antibody IgG | Cell Signaling Technology, USA |
| Rat anti-CD44 antibody IgG | Abcam, USA |
| Mouse anti-ceramide antibody IgM | Glycobiotech, Germany |
| FITC-phalloidin | Sigma, USA |
| Fluorescent secondary antibodies | Jackson ImmunoResearch, USA |
| Mouse anti-RhoA antibody | Cytoskeleton inc., USA |
| Mouse anti-Rac1 antibody | Cytoskeleton inc., USA |
| Mouse anti-Rac1 antibody | Cytoskeleton inc., USA |
| Beta-actin | Santa Cruz, USA |

**2.3 Tissue culture materials**

| | |
|---|---|
| MEM | Gibco/Invitrogen, Karlsruhe |
| Fetal Calf Serum (FCS) | Gibco/Invitrogen, Karlsruhe |
| L-Glutamine | Gibco/Invitrogen, Karlsruhe |
| Penicilin/Streptomycin | Gibco/Invitrogen, Karlsruhe |
| Sodium pyruvate | Gibco/Invitrogen, Karlsruhe |

**2.4 Equipments**

| | |
|---|---|
| Cell culture incubator | ThermoFisher Scientific, MA, USA |
| Cell culture flask | Corning Inc., NY, USA |
| Cell culture, 6, 24 and 96 well plate | Corning Inc., NY, USA |
| Cell scraper | TPP, Trasadingen, Switzerland |
| Cell strainer | Becton Dickinson Labware, France |
| Conical centrifuge tubes | BD Falcon, USA |
| Cover slips | Carl-Roth GmbH & Co, Karlsruhe |
| Cuvettes | Sarstedt, Nümbrecht, Germany |
| Hybond ECL nitrocellulose membrane | GE Healthcare, USA |
| Leica DMI-4000 fluorescence microscope | Leica Microsystem, Mannheim, Germany |
| Leica TCS SP5 confocal microscope | Leica Microsystem, Mannheim, Germany |
| Microscopic slides | Engelbrecht Medizin und labortechnik |

| | |
|---|---|
| | GmbH, Germany |
| Parafilm | Peckiney, Chicago, IL, USA |
| Rotary agitator | Neolab Migge Laborbedarf-Vertriebs GmbH,Germany |
| Silica G60 TLC plates | Merck, Darmstadt, Germany |
| SpeedVac | ThermoFisher Scientific, MA, USA |
| Thermomixer | Eppendorf, Germany |
| Typhoon FLA 9500 laser scanner | GE Healthcare Life Sciences, USA |
| X-ray films | FUJIFILM Medical Systems, USA |

**2.5 Buffers**

| | |
|---|---|
| Agarose gel (0.8%) | 0.8 g agarose |
| | 100 ml TAE buffer |
| Alcaline phosphatase wash buffer | 100 mM Tris/HCl pH 9.5 |
| | 100 mM NaCl |
| Anesthesia cocktail | 10% Ketamin 2 ml |
| | 2% Xylazin 0.5 ml |
| | $ddH_2O$ 10 ml |
| HEPES | 132 mM NaCl |
| | 20 mM Hepes pH 7.4 |
| | 5 mM KCl |
| | 1 mM $CaCl_2$ |
| | 0.7 mM $MgCl_2$ |
| | 0.8 mM $MgSO_4$ |
| Mowiol | 6 g Glycerol |
| | 2.4 g Mowiol |
| | 6 ml $ddH_2O$ |
| | 12 ml 0.2 M Tris-Base, pH 8.5 |
| | 0.1% DABCO |
| Phosphate buffered saline (PBS), pH 7.4 | 137 mM NaCl |

| | |
|---|---|
| | 2.7 mM KCl |
| | 10 mM $Na_2HPO4$ |
| | 1.8 mM $KH_2PO4$ |
| PBS-T | 137 mM NaCl |
| | 2.7 mM KCl |
| | 10 mM $Na_2HPO4$ |
| | 1.8 mM $KH_2PO4$ |
| | 0.05 % Tween 20 |
| Running buffer | 25 mM Tris |
| | 192 mM glycine |
| | 0.1 % SDS |
| Sample buffer (5X) | 250 mM Tris pH 6.8 |
| | 20 % Glycine |
| | 4 % SDS |
| | 8 % β-mercaptoethanol |
| | 0.2 % bromophenol blue |
| TBS-T | 20 mM Tris |
| | 150 mM NaCl |
| | 0.05 % Tween 20 |
| Transfer buffer | 10 mM $NaHCO_3$ |
| | 3 mM $Na_2CO_3$ |
| | 10 % Methanol |
| Trypsin | 0.25% Trypsin |
| | 5 mM Glucose |
| | 1.3 mM EDTA |

# 3 Methods

## 3.1 Mice and cells

Acid sphingomyelinase (Asm)-deficient mice and wild-type (WT) littermates (Horinouchi et al., 1995) (sphingomyelin phosphodiesterase 1 knockout; *Smpd1*$^{-/-}$) and CD44-deficient mice and wild type littermates WT mice (Olaku et al., 2011; Orian-Rousseau et al., 2002) were maintained on a C57BL/6J background. The genotype was verified by polymerase chain reaction (PCR). We used Asm-deficient mice and WT littermates aged 6 to 8 weeks to avoid sphingomyelin accumulation (Carpinteiro et al., 2015). Mice were bred in the animal facility of the University of Duisburg-Essen under specific pathogen-free (SPF) conditions according to the criteria of the Federation of Laboratory Animal Science. All procedures performed on mice were approved by the Bezirksregierung Düsseldorf, Düsseldorf, Germany.

The culture of bone marrow-derived macrophages (BMDMs) has been previously described in detail (Zhang et al., 2011). Briefly, mice were sacrificed, femurs and tibias were flushed with minimum essential medium (MEM; Gibco, UK) supplemented with 10% fetal bovine serum (Gibco), 10 mM HEPES (Roth GmbH; pH 7.4), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 µM non-essential amino acids, 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco). The samples were passed through a 23G needle to obtain single cells and cultured for 24 h in small tissue-culture flasks. Cells were washed and 3 × $10^4$ or 1.5 × $10^5$ non-adherent cells were cultured in 24- or 6-well plates in MEM with 20% L-cell supernatant as a source of macrophage colony-stimulating factor. Fresh MEM/L-cell supernatant media was applied after 4 days of culture. Macrophages mature within the next 6 days and were used on day 10 of culture.

Alveolar macrophages (AMs) were isolated from bronchoalveolar lavage (BAL) fluid (Zhang et al., 2011). Mice were sacrificed; the trachea was exposed, catheterized, and lavaged with a total of 15 mL phosphate-buffered saline (PBS). Cells were centrifuged for 5 min at 300 × g at 4$^{o}$C, supernatants were discarded, and the pellets were resuspended in MEM/HEPES. Cells were then seeded for further experiments.

### 3.2 Infection experiments

The *S. aureus* strain used in the present study was isolated from a patient with sepsis. The strain produces alpha toxin and enterotoxin D but not the Panton-Valentine leukocidin or the toxic shock syndrome toxin (Peng et al., 2015). *S. aureus* was grown overnight on trypticase soy agar (TSA) plates with 5% sheep's blood (BD), resuspended in 40 mL tryptic soy broth (BD) at an optical density of 0.2 to 0.25, and incubated at 37°C with shaking at 125 rpm for 75 min. Bacteria were pelleted by centrifugation at 2800 rpm for 10 min and washed with RPMI 1640 (Gibco) supplemented with 10 mM HEPES (Roth GmbH). The bacteria were finally resuspended in HEPES/Saline (H/S) buffer consisting of 132 mM NaCl, 20 mM HEPES (pH 7.4), 5 mM KCl, 1 mM $CaCl_2$, 0.7 mM $MgCl_2$, and 0.8 mM $MgSO_4$. Cells or mice were then infected within the next 10 min.

To determine Asm activity, ezrin/radixin/moesin (ERM) phosphorylation, and Rho family GTPase activity, we infected approximately $10^6$ macrophages in a 6-well plate with *S. aureus* at a multiplicity of infection (MOI) of 100 bacteria per macrophage. For all immunofluorescence studies or intracellular bacteria killing assay *in vitro*, macrophages were cultured in 24-well plates, and cells were infected with *S. aureus* at an MOI of 100 bacteria per BMDM or an MOI of 50 bacteria per AM. Macrophages were left uninfected or were infected with *S. aureus* indicated times below.

For *in vivo* experiments, we infected mice intranasally with $8 \times 10^8$ colony-forming units (CFUs) per mouse (Grassme et al., 2000). The mice were observed for 5 days or were sacrificed at 6 h or 12 h to determine the number of CFUs in the lungs.

To perform bacterial killing and phagocytosis assays *in vitro*, *c*ells were infected as described above. The infection was terminated by gently washing the cells with sterile PBS followed by incubation of the macrophages with or without 100 µg/mL gentamycin (Sigma) for 1 h at 37°C in MEM/HEPES to determine the number of internalized bacteria or the total number of intra- and extracellular bacteria. The macrophages were then extensively washed, lysed in 5 mg/mL saponin dissolved in PBS for 10 min to release intracellular bacteria, pelleted by centrifugation at 3200 rpm, resuspended in

PBS, aliquots were plated on Luria broth (LB)-agar plates, and the CFUs were counted after growth overnight at 37°C.

For *in vivo* assays mice were infected with *S. aureus* and sacrificed at 6 h or 12 h for detecting total bacteria in the lung. The lung tissue was collected, homogenized into very small pieces, lysed with 5 mg/mL saponin/PBS, and total bacteria were determined as described above. Mice were sacrificed at 6 h and lung homogenates were incubated with 100 µg/mL gentamycin in PBS for 1 h or left untreated for detecting intracellular bacteria in the lung. The samples were then extensively washed. Next, the lung tissue was lysed with 5 mg/mL saponin/PBS, and the CFU of internalized were determined as described above.

### 3.3 Assay for acid sphingomyelinase activity

Acid sphingomyelinase activity was measured with green fluorescent BODIPY-$FL_{C12}$-sphingomyelin (Thermo Fisher Scientific) as a substrate. Briefly, cells were infected or left untreated, harvested and lysed in 250 mM sodium acetate (Sigma) and 1% Nonidet P-40 (pH 5.0; Sigma) for 5 min on ice. Cells were further disrupted by sonification for 10 min in an ice bath sonicator (Bandelin Electronic). The protein concentration was measured by a Bradford protein assay (BioRad), and 5 µg of protein in 20 µL lysis buffer was added to 250 mM sodium acetate (pH 5.0) containing 100 pmol BODIPY-$FL_{C12}$-sphingomyelin. The samples were incubated at 37°C for 1 h with shaking at 300 rpm. The reaction was stopped by the addition of 1 mL chloroform:methanol (2:1, v/v) followed by centrifugation for 5 min at 14 000 rpm. The lower phase was dried in a SpeedVac Concentrator (Thermo Fisher Scientific) and resuspended in chloroform:methanol (2:1, v/v). The samples were spotted on a thin-layer chromatography (TLC) plate (Merck, Germany), separated with chloroform:methanol (80:20, v/v), scanned with a Typhoon FLA 9500 laser scanner (GE Healthcare Life Sciences, USA), and analyzed with ImageQuant software (GE Healthcare Life Sciences).

### 3.4 Immunocytochemistry

Macrophages were grown on coverslips and were left uninfected or infected with *S. aureus*. Infection was stopped by adding 4% paraformaldehyde (PFA; Sigma) in PBS (pH 7.4) for 10 min. Cells were washed 3-times with H/S. For confocal microscopy macrophages were permeabilized with 0.1% Triton X-100/PBS for 5 min at room temperature. Cells were washed again with PBS and incubated for 1 h with goat anti-Armenian hamster immunoglobulin G (IgG) antibodies (dilution 1:100; Jackson ImmunoResearch) to block non-specific binding. Samples were washed and incubated for 1 h with rabbit monoclonal IgG anti-phospho-ezrin (phospho Thr567)/anti-phospho-radixin (phospho Thr564)/anti-phospho-moesin (phospho Thr558) antibodies (1:200; Cell Signaling Technology), rat monoclonal IgG2b anti-CD44 antibodies (1:200 diluted; Abcam), mouse monoclonal IgM anti-ceramide antibodies (1:100 diluted; Glycobiotech), rabbit polyclonal IgG anti-*S. aureus* antibody (1:200 diluted), mouse monoclonal IgG anti-*S. aureus* antibody (1:200 diluted), or fluorescein isothiocyanate-conjugated (FITC) phalloidin (25 µg/mL; Sigma). All antibodies were diluted in 5% FCS/PBS. Cells were then washed 3-times for 5 min each with 0.05% Tween-20/PBS and incubated with secondary antibodies corresponding to the primary antibodies: FITC-conjugated $F(ab')_2$ fragments of goat anti-rat IgG; Cy3-conjugated $F(ab')_2$ fragments of donkey anti-rat IgG, donkey anti-mouse IgM, and donkey anti-rabbit IgG; DyLight 649-conjugated $F(ab')_2$ fragments of donkey anti-mouse IgG; and Alexa Fluor 647-conjugated $F(ab')_2$ fragments of donkey anti-rabbit IgG for 45 min (all antibodies from Jackson ImmunoResearch; final concentration of all antibodies, 1.5 µg/mL; diluted in 5% FCS/PBS). The samples were then washed 3-times with PBS/0.05% Tween 20 and once in PBS and were then mounted with Mowiol (Kuraray Specialities Europe GmbH, Germany). Samples were visualized with a Leica TCS SP5 confocal microscope using a 100× oil lens, and images were analyzed with Leica LCS software (Leica Microsystems).

For scanning electron microscopy, macrophages were treated as above. After fixation and washing with H/S cells were dehydrated in graded series of ethanol followed by critical-point drying (CPD 7501; Polaron) and were then sputtered with platinum/palladium (208HR high-resolution sputter coater; Cressington). Specimens

were analyzed with an S-4000 scanning electron microscope (SEM) (Hitachi), and images were obtained with a DISS5 (Point Electronics) analysis system.

### 3.5 Western blots and pull-down assay

Cells were lysed in 100 µL 0.1% sodium dodecyl sulfate (SDS), 25 mM HEPES, 0.5% deoxycholate, 0.1% Triton X-100, 10 mM ethylenediaminetetraacetic acid (EDTA), 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 125 mM NaCl, and 10 µg/mL aprotinin/leupeptin (Sigma). Samples were centrifuged at 14 000 rpm for 5 min at 4°C, and supernatants were collected. Proteins were separated by 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes, followed by blocking with 5% bovine serum albumin (BSA) in Tris-buffered saline supplemented with 0.05% Tween 20. Blots were incubated overnight at 4°C with anti-phospho-ERM (1:2000; Cell Signaling Technology) or anti-Ezrin antibodies (1:1000; Cell Signaling Technology). Blots were washed and developed with alkaline phosphatase-coupled secondary antibodies (1:20,000; Santa Cruz Biotechnology Inc.) using the Tropix chemoluminescence system (Amersham Pharmacia Biotech Inc.).

Rho family GTPase activity was detected using the RhoA/Rac1/Cdc42 Activation Assay Combo Biochem Kit (Cytoskeleton Inc.) according to the manufacturer's instructions. Briefly, cells were infected and lysed in 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 0.5 M NaCl, and 2% Igepal. Equivalent amounts of protein were added to a pre-determined amount of rhotekin Rho binding domain (for RhoA activation assay) or PAK-PBD beads (for Rac1 and Cdc42 activation assays) and incubated at 4°C on a rotator for 1 h. Beads were washed with a buffer consisting of 25 mM Tris (pH 7.5), 30 mM $MgCl_2$, and 40 mM NaCl. Finally, 20 µL of Laemmli sample buffer were added to each sample. Proteins were separated on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. Blots were incubated with anti-RhoA monoclonal antibody (ARH04), anti-Rac1 monoclonal antibody (ARC03), or anti-Cdc42 monoclonal antibody (ACD03) (all antibodies from Cytoskeleton Inc.) as described by the vendor and developed as described above.

### 3.6 Phagosome-lysosome-fusion

For detection of phagosome-lysosome fusion, macrophages were pre-incubated with 1 mg/mL tetramethylrhodamine isothiocyanate (TMR) dextran (10 kD, Sigma-Aldrich) for 1 h in HEPES/MEM at 37°C. Cells were then washed with HEPES/MEM and infected with *S. aureus* as above. Cells were fixed and permeabilized as above. Lysosomes were stained with an anti-lysosome-associated membrane protein 1 (Lamp1) antibody (Abcam) followed by incubation with FITC-coupled anti-rat antibodies. For observation of intracellular acidic organelles, macrophages were incubated with 60 nM LysoTracker Red DND-99 or 1 µM LysoSensor DND-189 (ThermoFisher) in MEM. Macrophages were then infected with *S. aureus*, washed, and analyzed with confocal microscopy, as described above.

### 3.7 Statistics

Data are expressed as arithmetic means ± standard deviation (SD) unless otherwise indicated. One-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test was used to test between-group and within-group differences; pairwise comparisons were performed with Student's *t*-test. Comparisons of survival variables were performed with log-rank tests. Statistical significance was set at the level of $p < 0.05$. All data were obtained from independent measurements. The GraphPad Prism statistical software program (GraphPad Software, USA) was used for the analyses.

# 4 Results

## 4.1 Acid sphingomyelinase is activated upon *S. aureus* infection and is crucially involved in internalization of the pathogen

The engulfment of bacteria into macrophages is a crucial mechanism of the host-defense system for eliminating pathogens, but it is also a mechanism used by the bacteria to escape the hostile extracellular environment and the immune system and thus to survive in infected cells. To determine whether Asm is involved in the uptake of *S. aureus*, we infected WT and Asm-deficient BMDMs with *S. aureus* for 30, 60, or 120 min or left them uninfected and then determined Asm activity. The results show a rapid and strong activation of Asm in WT macrophages but no Asm activity in Asm-deficient macrophages (Fig. 4.1A and B). Wild-type macrophages rapidly internalized *S. aureus*, which was severely reduced in Asm-deficient macrophages (Fig. 4.1C). Asm deficiency did not affect the adhesion of *S. aureus* bacteria to macrophages (Fig. 4.1D). Taken together, these findings indicate that Asm is activated by and is crucial for *S. aureus* internalization in BMDMs.

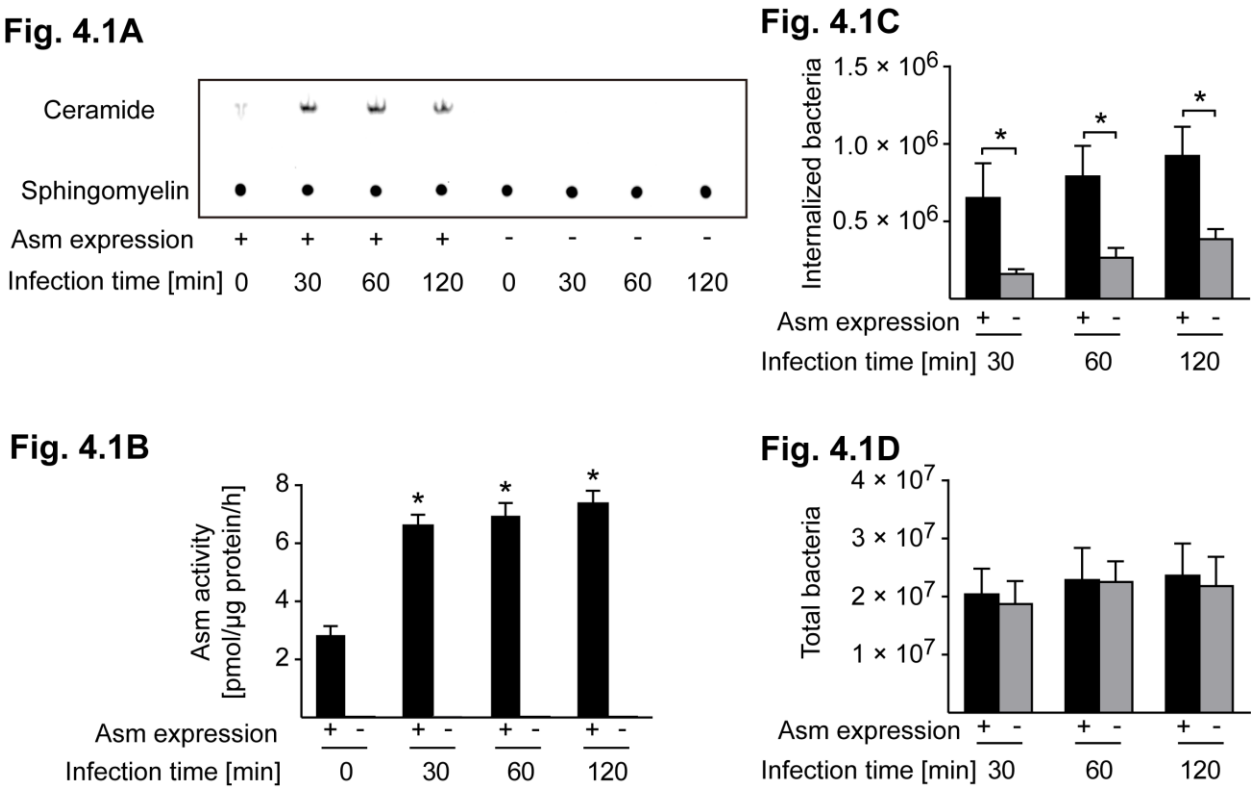



**Fig. 4.1: Staphylococcus aureus infection activates acid sphingomyelinase that mediates internalization of Staphylococcus aureus into macrophages**

**(A)** Bone marrow-derived macrophages (BMDMs) were infected and lysed, and the activity of acid sphingomelinase (Asm) was determined by the consumption of BODIPY-FLC12-sphingomyelin. Samples were extracted and separated on thin-layer chromatography (TLC) plates, which were then scanned with a Typhoon laser scanner. Panel A shows a representative result. Panel **B** shows the mean ± standard deviation (SD) of 3 independent experiments using ImageQuant, *p < 0.05, t-test. **(C, D)** Wild-type (WT) and acid sphingomyelinase (Asm)-deficient bone marrow-derived macrophages (BMDMs) were infected with S. aureus for 30, 60, or 120 min or left uninfected. The colony forming units (CFUs) of internalized **(C)** or total number (extra- and intracellular) bacteria **(D)** were determined. Data are shown as mean ± SD of 3 independent experiments, *p < 0.05, one way ANOVA followed Student-Newman-Keuls test.

## 4.2 Acid sphingomyelinase is required for internalization induced actin polymerization

Actin cytoskeleton dynamics in macrophages are essential for the recognition of pathogens and for phagocytosis (Kuwae et al., 2016; Man et al., 2014; Siegrist et al., 2015). Infection of WT BMDMs with *S. aureus* resulted in dramatic changes of the cell shape, as determined by scanning electron microscopy (SEM) (Fig. 4.2A). Filopodia began to form as early as 30 min after *S. aureus* infection of WT macrophages and continued to extend at 60 and 120 min after infection. The formation and extension of filopodia were much less prominent and occurred much more slowly in Asm-deficient macrophages infected with *S. aureus* (Fig. 4.2A). Immunofluorescence staining of BMDMs (Fig. 4.2B) or of AMs (Fig. 4.2C) with phalloidin confirmed these results and showed that actin filaments reassemble and aggregate at the cell surface in WT macrophages after infection with *S. aureus*, but not in Asm-deficient macrophages.

The ERM family of actin-binding proteins function as linkers between the plasma membrane and the actin cytoskeleton and transduce signals to mediate cytoskeleton remodeling (Fehon et al., 2010; Hamada et al., 2003). ERM proteins are activated by phosphorylation (Fukata et al., 1998; Nakamura et al., 1995; Ng et al., 2001). To determine whether Asm (indirectly) mediates the phosphorylation of ERM proteins upon *S. aureus* infection, we infected WT and Asm-deficient BMDMs with *S. aureus*. We then stained the cells with immunofluorescent anti-phospho-ERM antibodies or lysed them, performed Western blots, and measured ERM phosphorylation, respectively. *S. aureus* infection induced a rapid and marked phosphorylation of ERM proteins, whereas there was almost no induction of phosphorylation in Asm-deficient BMDMs or AMs (Fig. 4.2B-E). Phosphorylated ERM proteins (pERM) rapidly co-localized with phalloidin, newly formed filopodia, and cell-associated bacteria in WT macrophages (Fig. 4.2B and C); in contrast, there were almost no changes of pERM in Asm-deficient BMDMs or AMs (Fig. 4.2B and C).

**Fig. 4.2A**

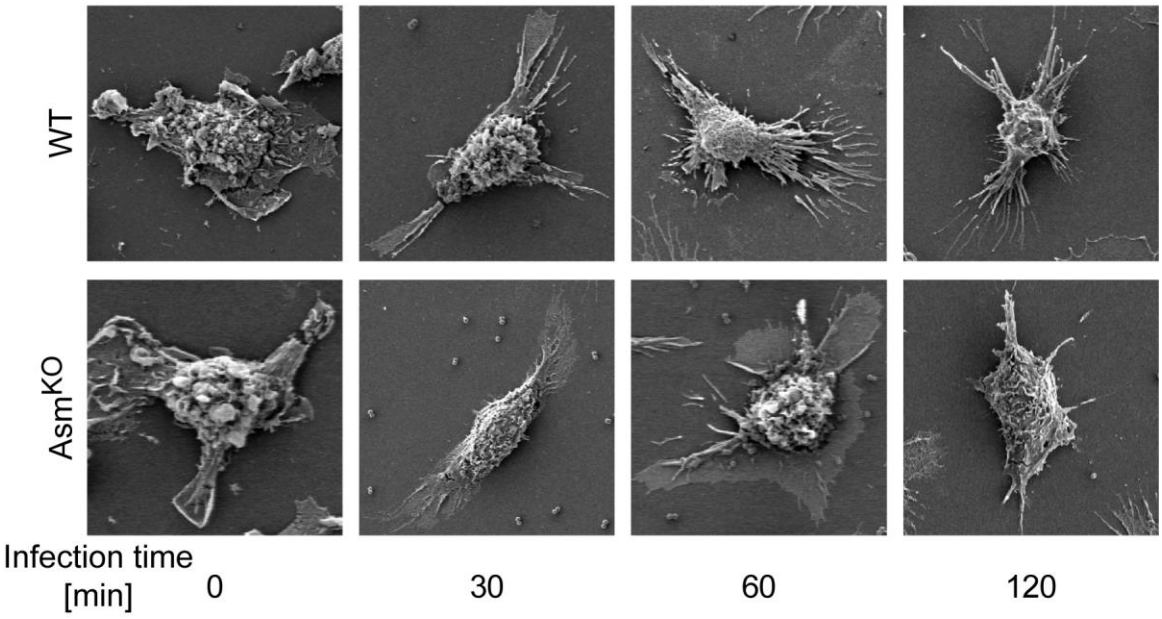



**Fig. 4.2: Acid sphingomyelinase controls actin cytoskeleton rearrangement and phosphorylation of ezrin/radixin/moesin after *S. aureus* infection of macropahges**

(**A**) Wild-type (WT) and acid sphingomyelinase (Asm)-deficient bone marrow-derived macrophages (BMDMs) were left uninfected or infected with *S. aureus* and analyzed for cell morphology by scanning electron microscopy (SEM). Shown are representative images from 3 independent experiments. B-E is shown in next pages. (**B, C**) Immunofluorescence studies using fluorescein isothiocyanate (FITC) phalloidin show a marked change of the actin cytoskeleton upon infection of WT BMDMs (**B**) and alveolar macrophages (AMs) (**C**) with *S. aureus*, a change that is absent from cells lacking Asm. (**B-E**) Rearrangement of the actin cytoskeleton is accompanied with the phosphorylation of ezrin/radixin/moesin, as determined by confocal microscopy (**B, C**) or Western blot studies (**D, E**). An ezrin antibody was used to confirm similar loading of all lanes. Shown are representative results (**B-D**) and mean ± SD of quantifications of the Western blot results of three independent experiments. * $p < 0.05$, ANOVA followed by Student-Newman-Keuls test.

**Fig. 4.2B**

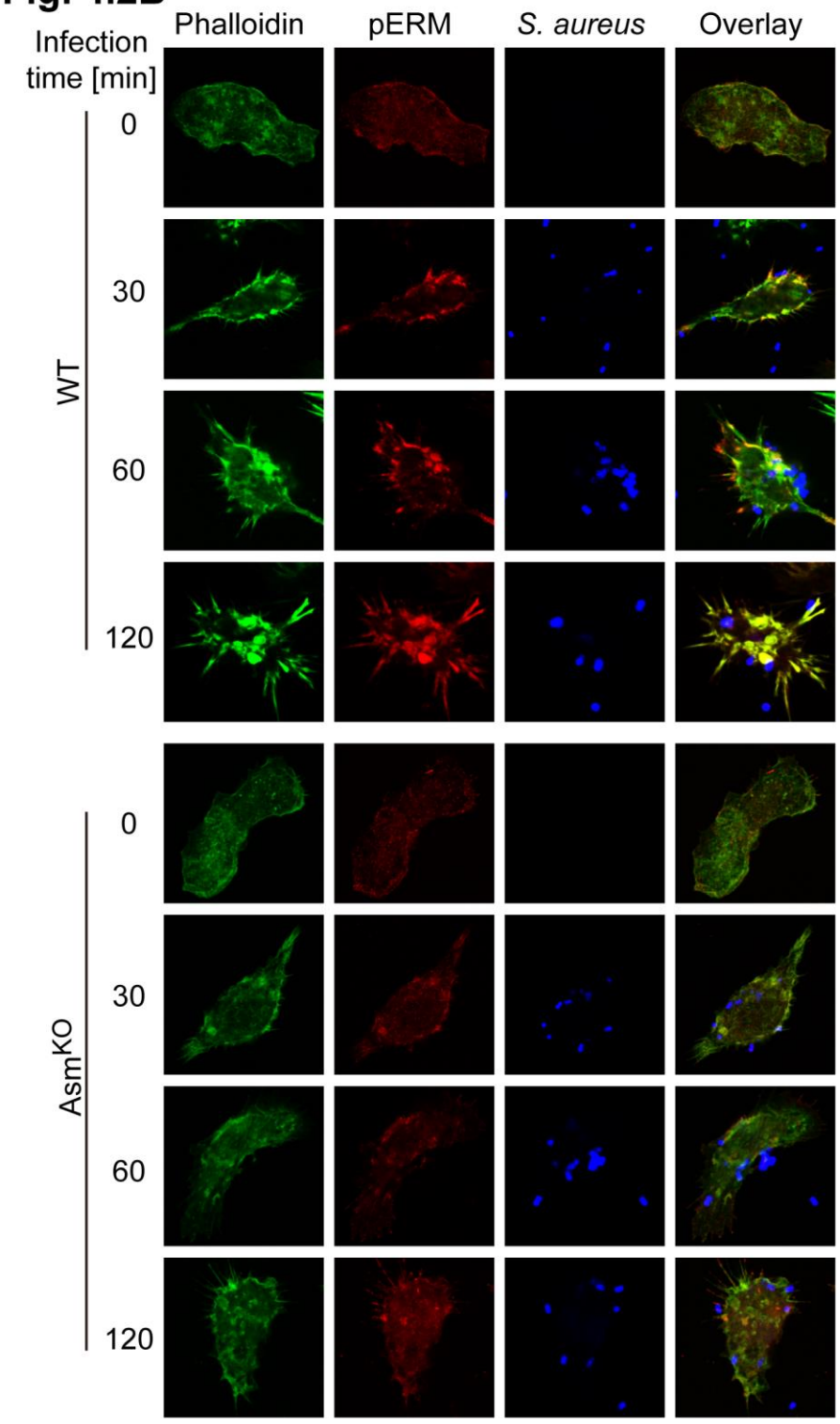

Phalloidin
pERM
S. aureus
Overlay
Infection time [min]
WT
0
30
60
120
AsmKO
0
30
60
120

**Fig. 4.2C**

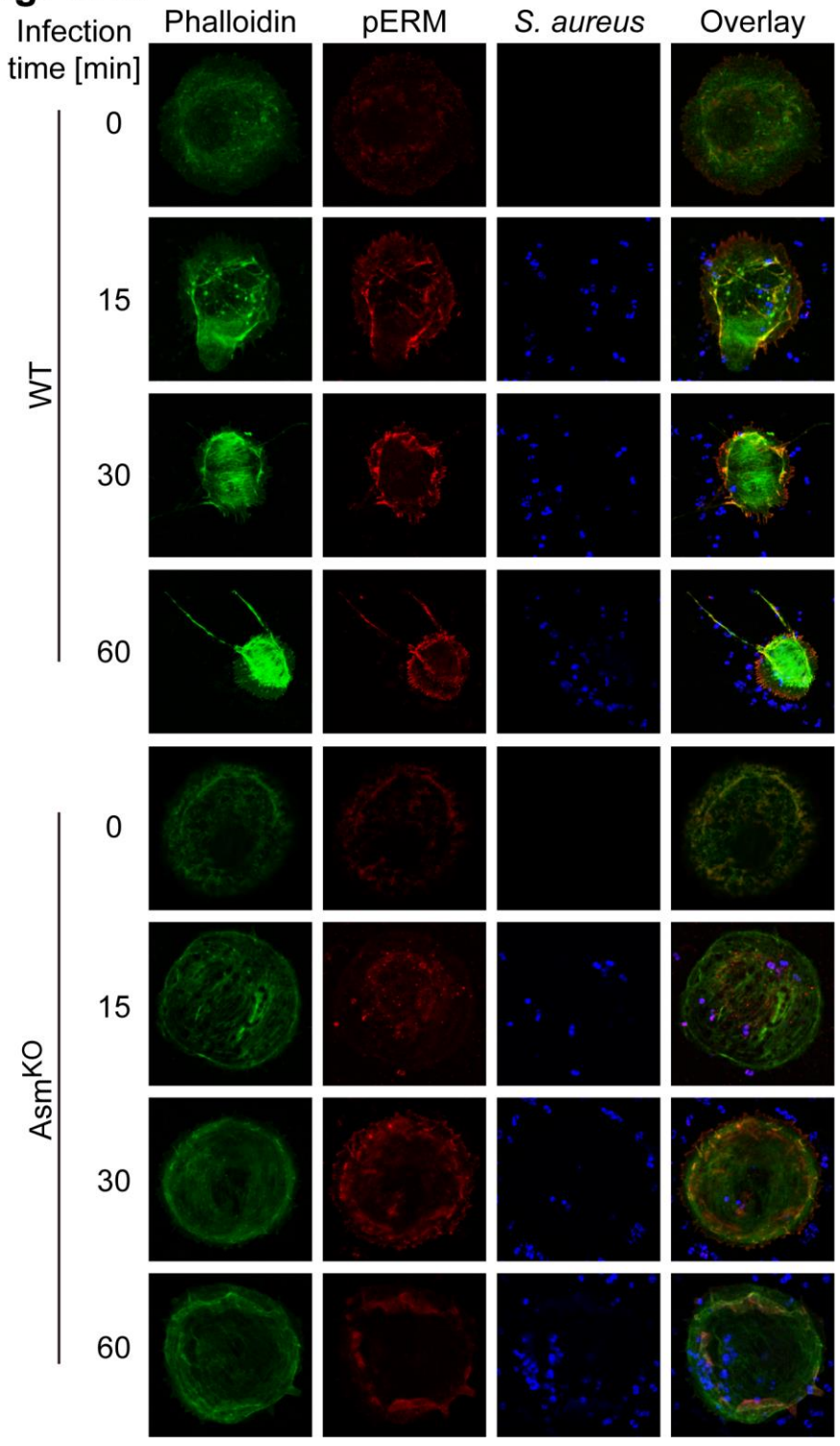

Infection time [min]
Phalloidin
pERM
S. aureus
Overlay
WT
0
15
30
60
AsmKO
0
15
30
60

**Fig. 4.2D**

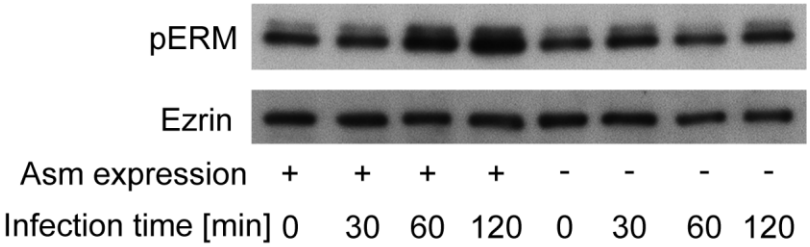
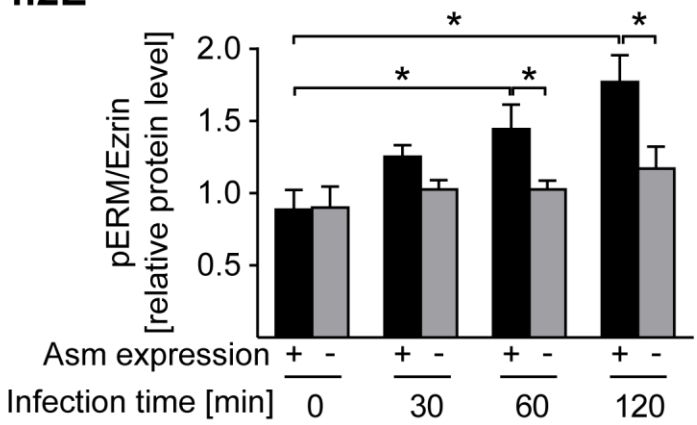



**Fig. 4.2E**




### 4.3 Acid sphingomyelinase regulates the activation of Rho GTPase upon *S. aureus* infection

RhoA, Rac1, and CDC42 are members of the Rho family of small GTPases, which regulate many aspects of intracellular actin dynamics (Ridley and Hall, 1992; Ridley et al., 1992; Schulz et al., 2015). To determine whether Asm regulates the activity of those Rho GTPases upon *S. aureus* infection, we determined their activity by pull-down assays. RhoA, Rac1, and Cdc42 were activated upon *S. aureus* infection in WT BMDMs (Fig. 4.3A-C), whereas their activation was almost completely abrogated by Asm deficiency (Fig. 4.3A-C).

Collectively, these findings indicate that Asm in macrophages is necessary for *S. aureus*-induced morphology changes, actin cytoskeleton rearrangement, ERM phosphorylation, and Rho GTPase activation.

**Fig. 4.3A**

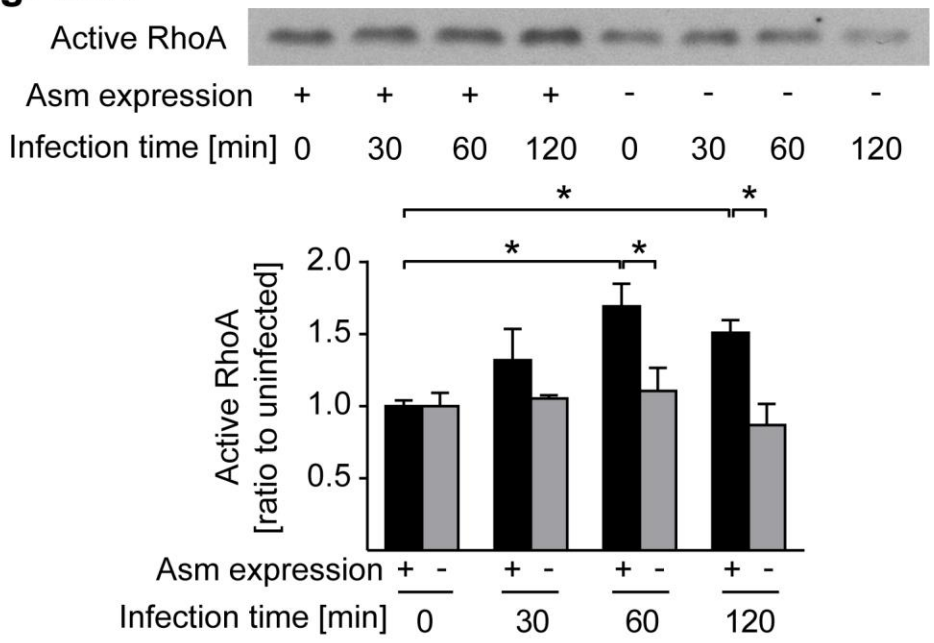
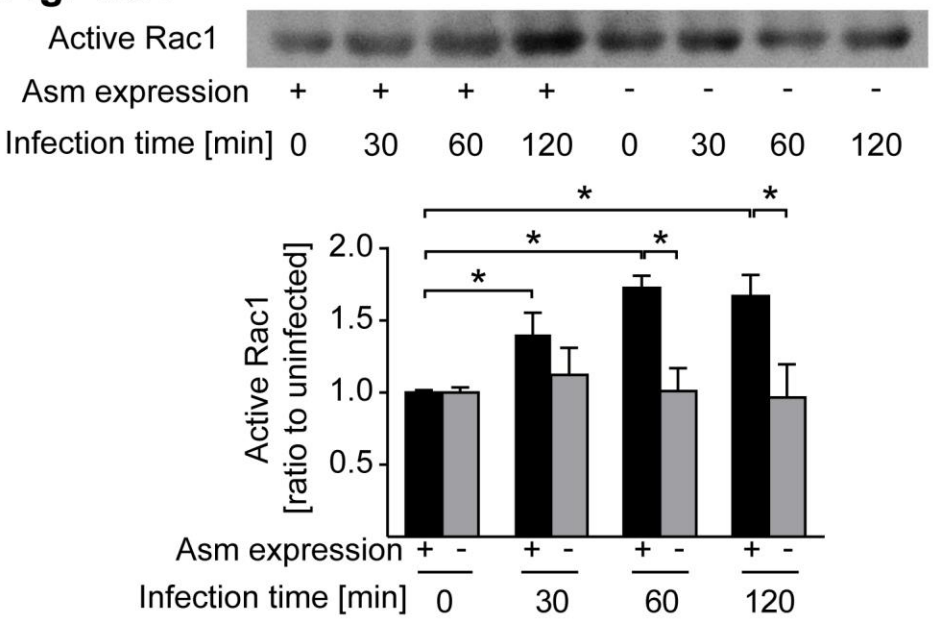



**Fig. 4.3B**



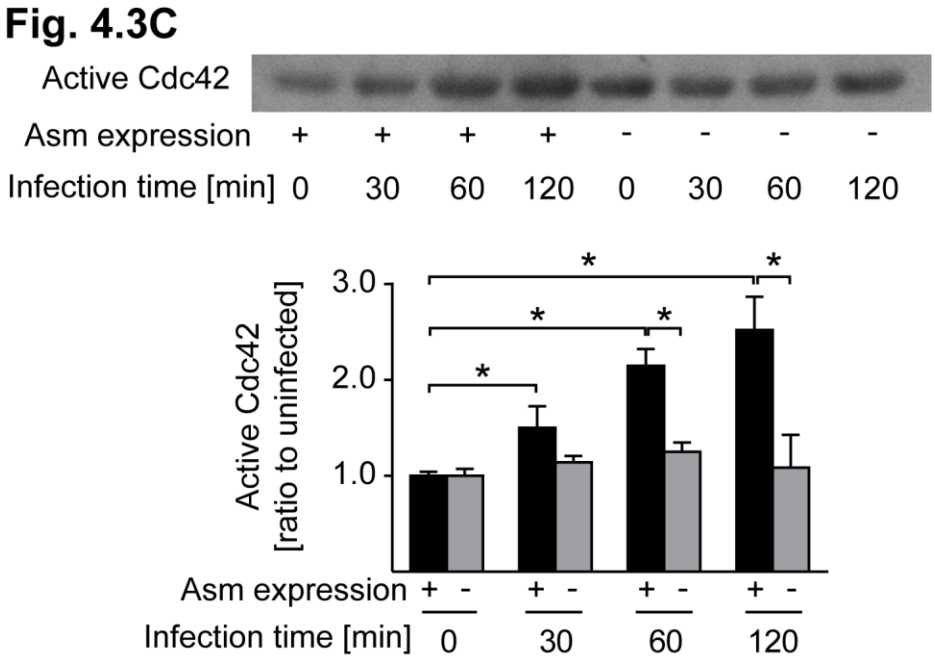



**Figure 4.3: Acid sphingomyelinase mediates activation of Rho family GTPases upon *S. aureus* infection of macrophages**

**(A-C)** Top: RhoA (A)/Rac1 (B)/Cdc42 (C) activity was determined by a pull-down assay from lysates obtained from S. aureus-infected or non-infected BMDMs. Bottom: quantification of the results of RhoA/Rac1/Cdc42 activity as determined by ImageJ. Values are means ± SD of 3 independent experiments; * p< 0.05, ANOVA followed by Student-Newman-Keuls test.

### 4.4 CD44 interacts with Asm in the infection process of *S. aureus*

CD44 is a glycoprotein that interacts with ERM and links the actin cytoskeleton to the plasma membrane and the extracellular matrix (Liu and Sy, 1997; Tsukita et al., 1994). We hypothesized that CD44 may serve as a receptor for *S. aureus*, linking the pathogen to the cytoskeleton and the Asm-ceramide signaling pathway. To study this hypothesis, we infected WT and Asm-deficient BMDMs with *S. aureus* and stained them with specific antibodies to CD44. The results reveal that infection with *S. aureus* leads to a translocation of CD44 to the plasma membrane and co-localization with actin filaments, which were recruited to the infection site at the cell surface. These translocation and co-localization events were almost completely absent in Asm-deficient BMDMs or AMs (Fig. 4.4A and C). Furthermore, upon infection with *S. aureus*, CD44 clustered within ceramide-enriched membrane platforms in WT BMDMs or AMs, events that were again absent in Asm-deficient cells (Fig. 4.4B and D).

**Figure 4.4: Acid sphingomyelinase regulates CD44 co-aggregation with phalloidin and ceramide**

Figures are shown in bellowing pages. Wild-type (WT) and acid sphingomyelinase (Asm)-deficient bone marrow-derived macrophages (BMDMs) **(A, B)** or alveolar macrophages (AMs) **(C, D)** were left uninfected or were infected with *S. aureus* for 30, 60, or 120 min or 15, 30, 60 min. Cells were then fixed and stained with anti-phalloidin, anti-CD44, and anti-*S. aureus* antibodies or with anti-CD44, anti-ceramide, and anti-*S. aureus* antibodies and corresponding fluorescent secondary antibodies. The samples were analyzed by confocal microscopy. Shown are representative results from 3 independent studies.

**Fig. 4.4A**

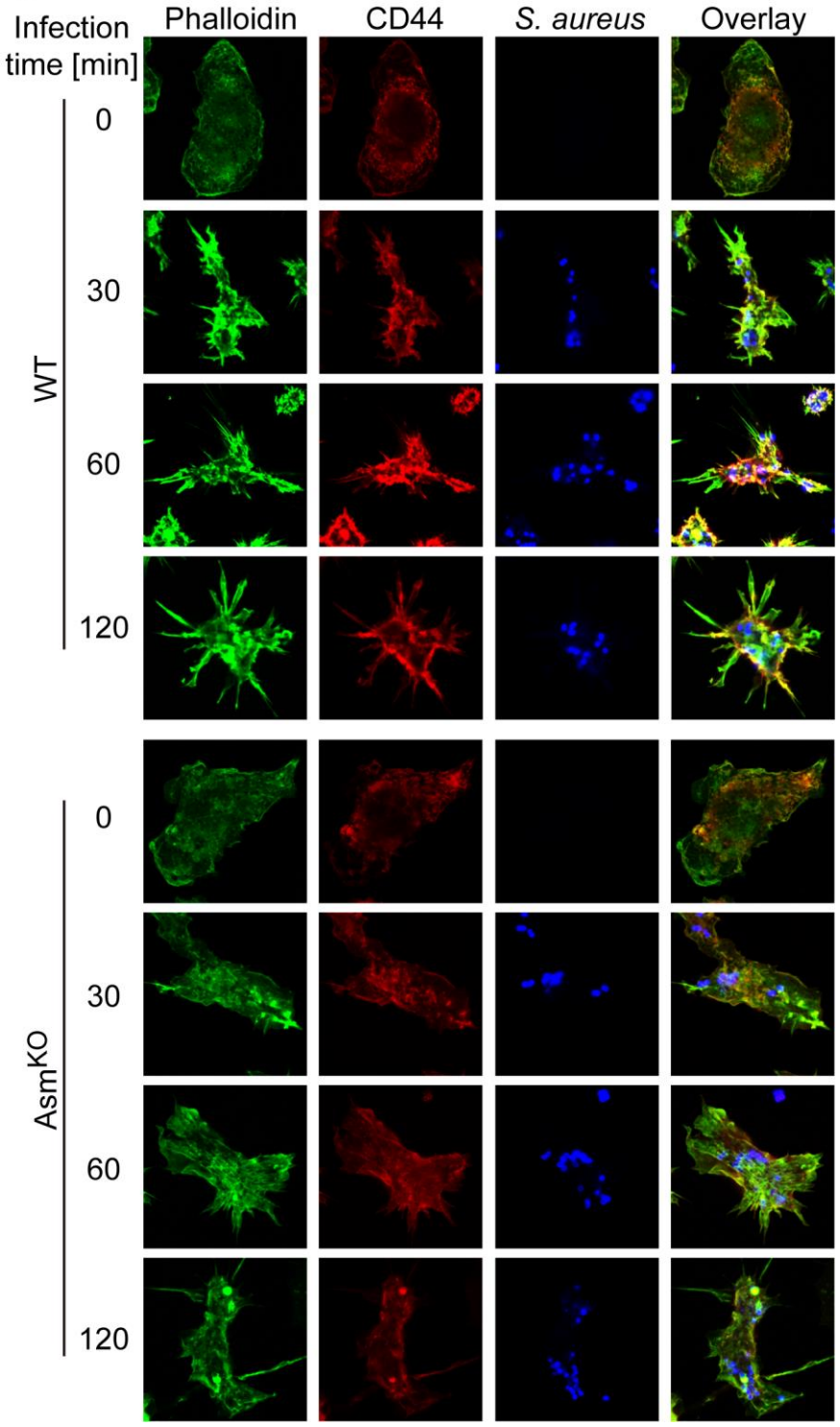

Infection time [min]
Phalloidin
CD44
S. aureus
Overlay
WT
0
30
60
120
AsmKO
0
30
60
120

**Fig. 4.4B**

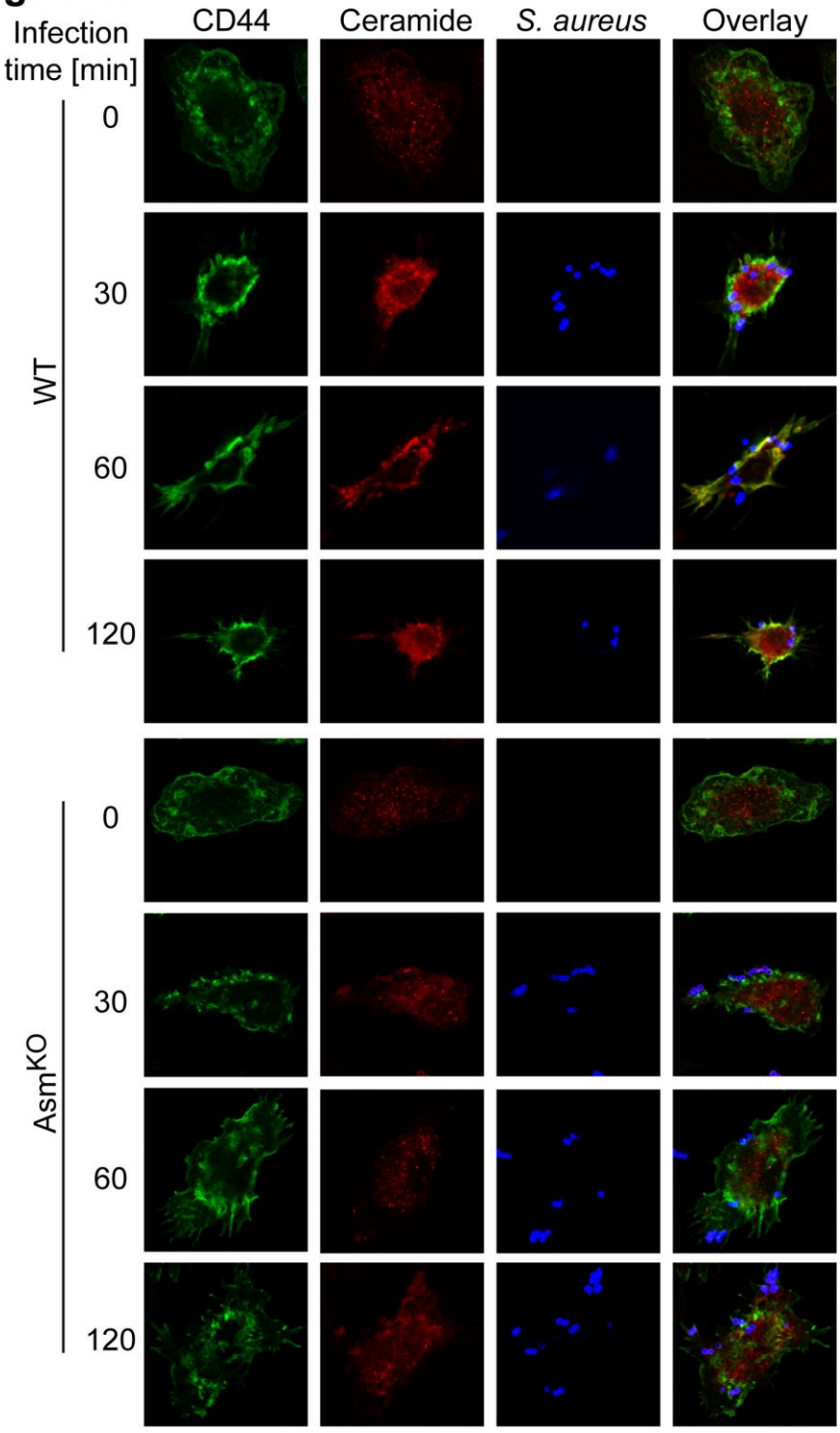

CD44
Ceramide
S. aureus
Overlay
Infection time [min]
WT
0
30
60
120
AsmKO
0
30
60
120

**Fig. 4.4C**

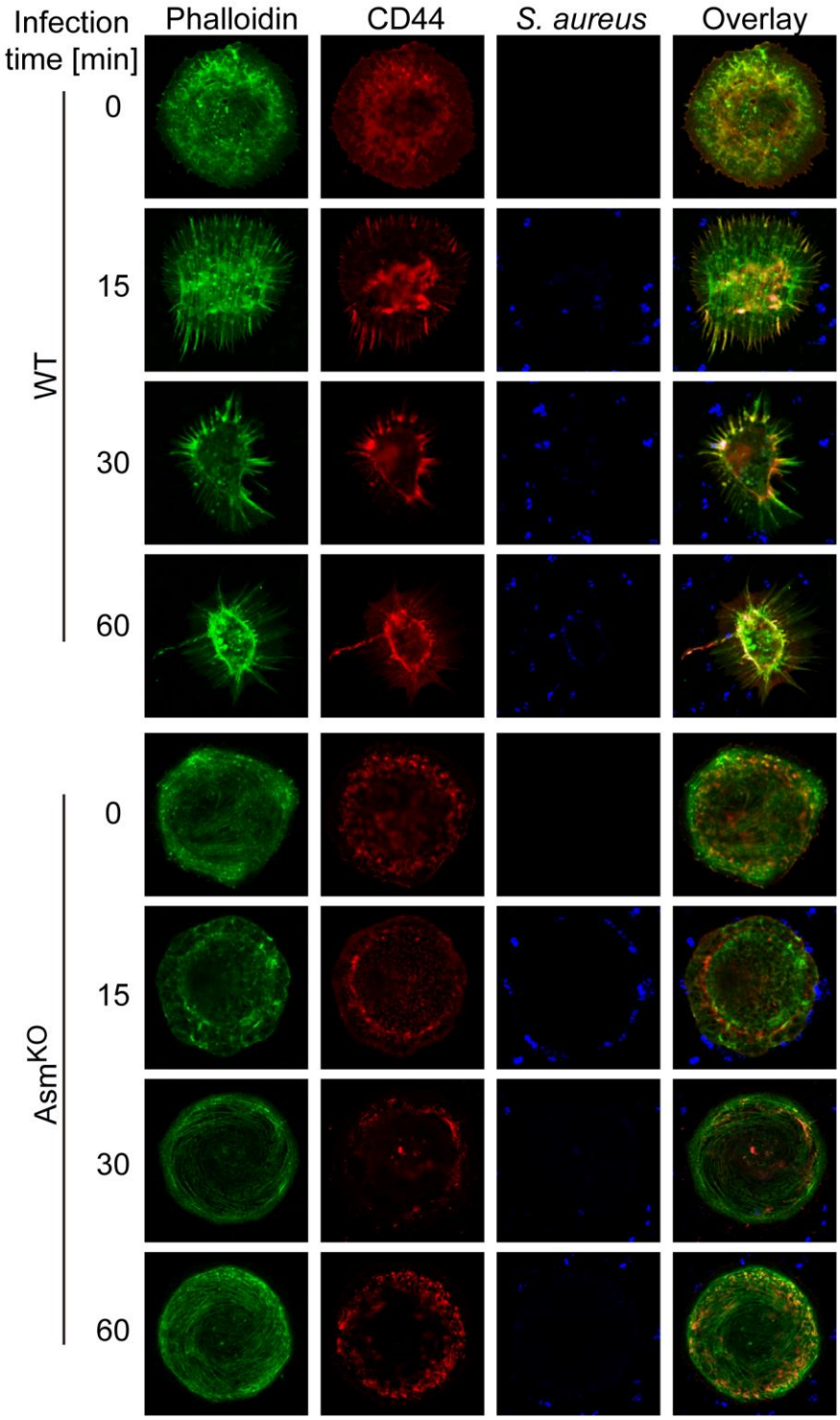
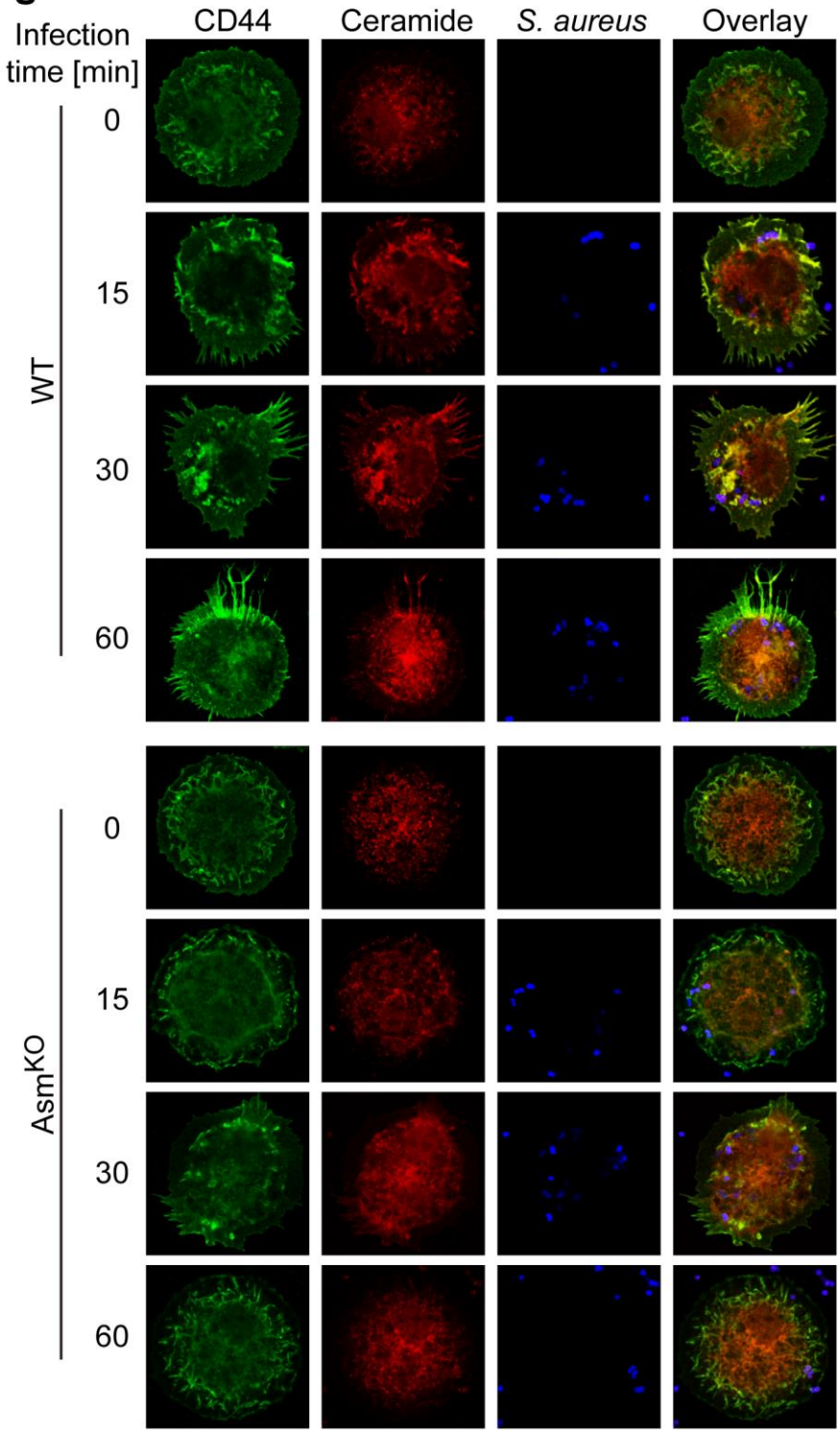

Infection time [min]
Phalloidin
CD44
S. aureus
Overlay
WT
0
15
30
60
AsmKO
0
15
30
60

**Fig. 4.4D**


CD44
Ceramide
S. aureus
Overlay
Infection time [min]
WT
0
15
30
60
AsmKO
0
15
30
60

### 4.5 *S. aureus* binds to CD44 which acts as upstream of Asm

To determine whether CD44 links to the Asm-ceramide system, we infected WT and CD44-deficient BMDMs with *S. aureus* and determined Asm activity. Our results show a marked activation of Asm after infection of WT macrophage with *S. aureus* (Fig. 4.5 and B). CD44 deficiency reduced and delayed the activation of Asm but did not completely prevent the stimulation of the enzyme (Fig. 4.5A and 5B).

These findings suggest that CD44 functions upstream of Asm. Therefore, we investigated whether CD44 is also involved in the uptake of *S. aureus*, the rearrangement of the cytoskeleton, and the phosphorylation of ERM proteins. The results show that CD44-deficiency in macrophages prevents *S. aureus* internalization, whereas adhesion is unaffected (Fig. 4.5C and 5D). Like Asm-deficient cells, *S. aureus*-infected CD44-deficient BMDMs did not rearrange the cytoskeleton to polymerize cortical actin, to cluster actin at ceramide-enriched membrane platforms, or to phosphorylate and translocate ERM proteins (Fig. 4.5E-G). Infection with *S. aureus* triggered a clustering of CD44 in ceramide-enriched membrane domains. Formation of these domains was abrogated by CD44 deficiency (Fig. 4.5H).

These findings strongly suggest that the Asm-ceramide system serves as a downstream target of CD44 during infection with *S. aureus* and mediates clustering of CD44 and thereby amplify signaling via the receptor.

**Fig. 4.5A**

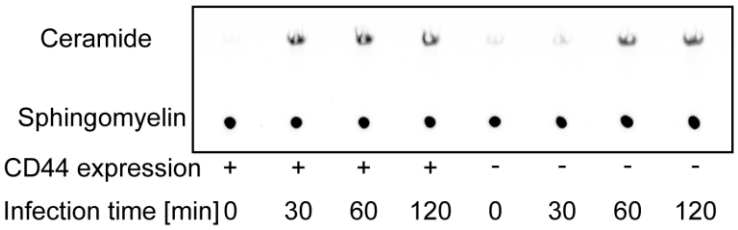



**Fig. 4.5C**

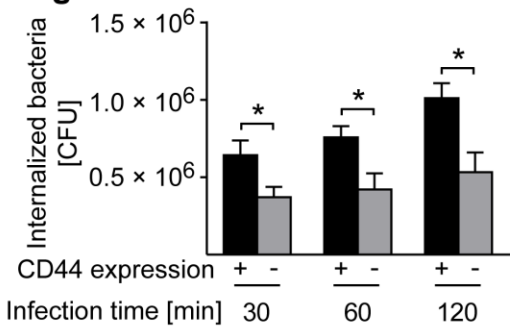



**Fig. 4.5B**

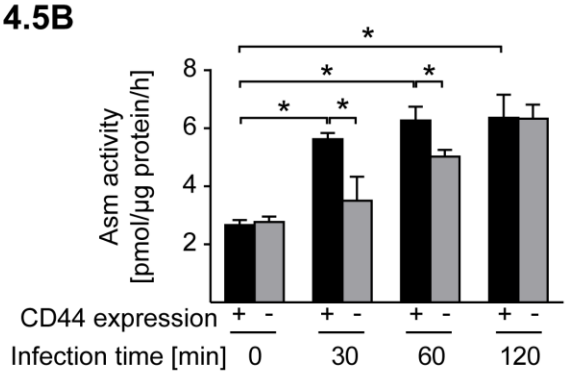



**Fig. 4.5D**

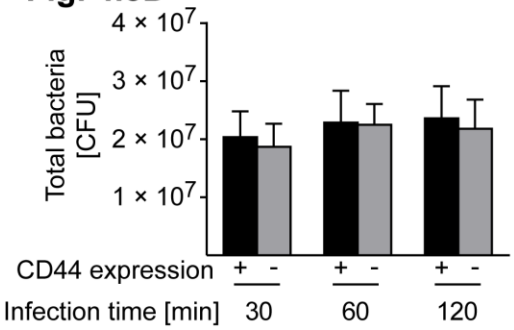



**Fig. 4.5E**

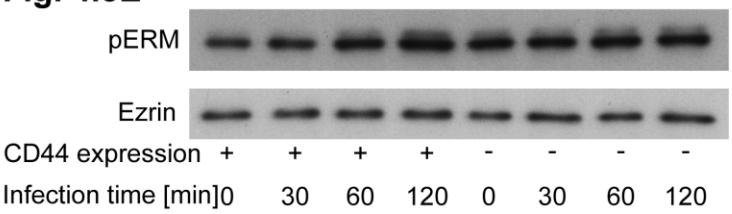



**Fig. 4.5F**

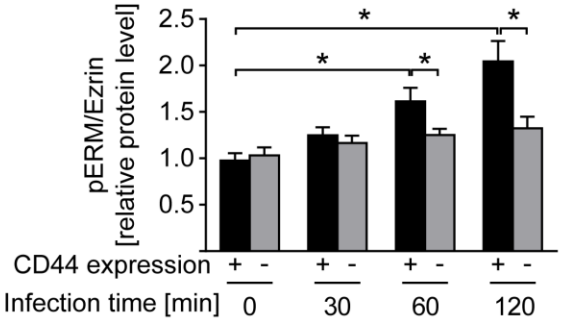


**Fig. 4.5G**

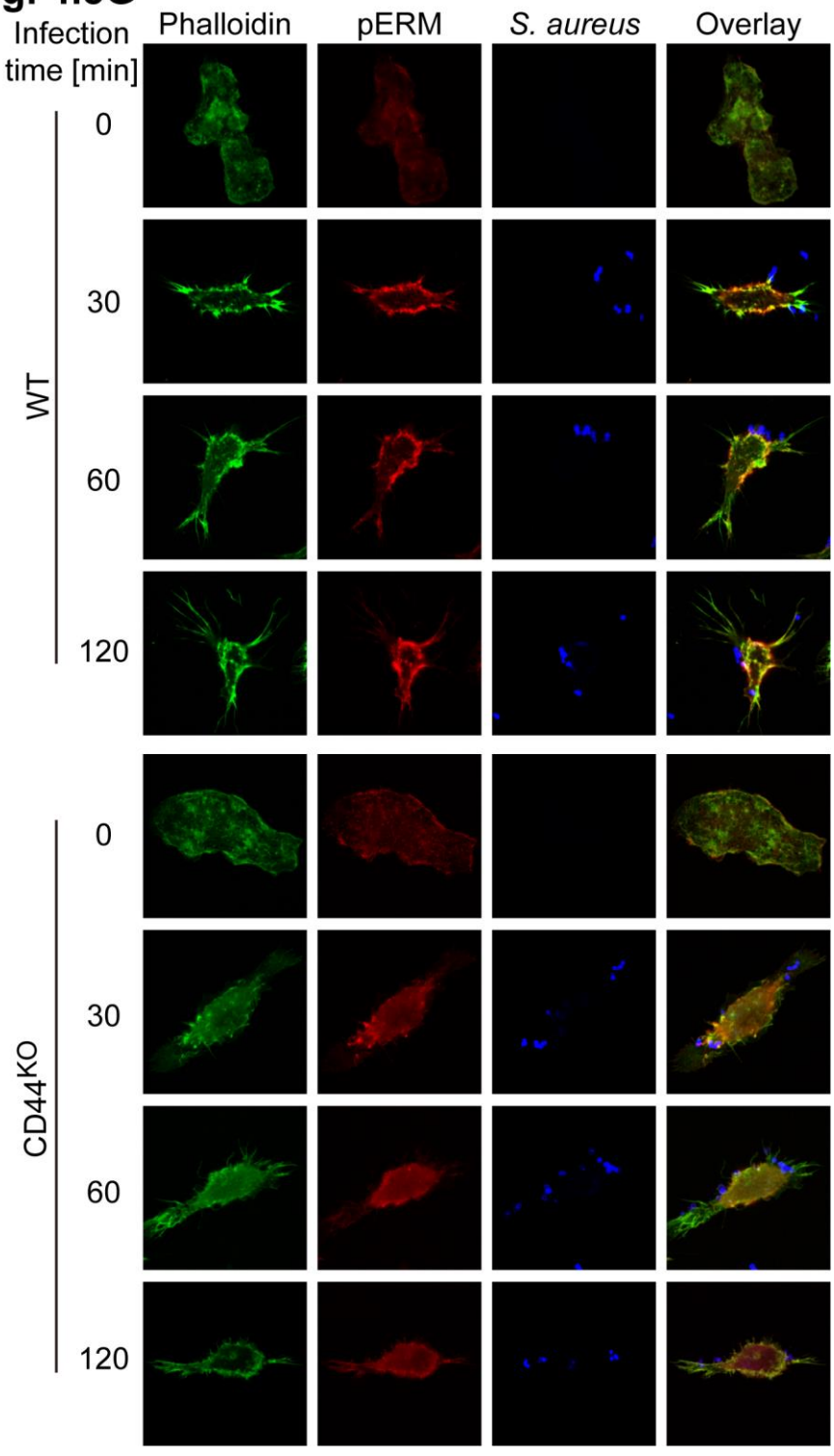

Infection time [min]
Phalloidin
pERM
S. aureus
Overlay
WT
0
30
60
120
CD44KO
0
30
60
120

**Fig. 4.5H**

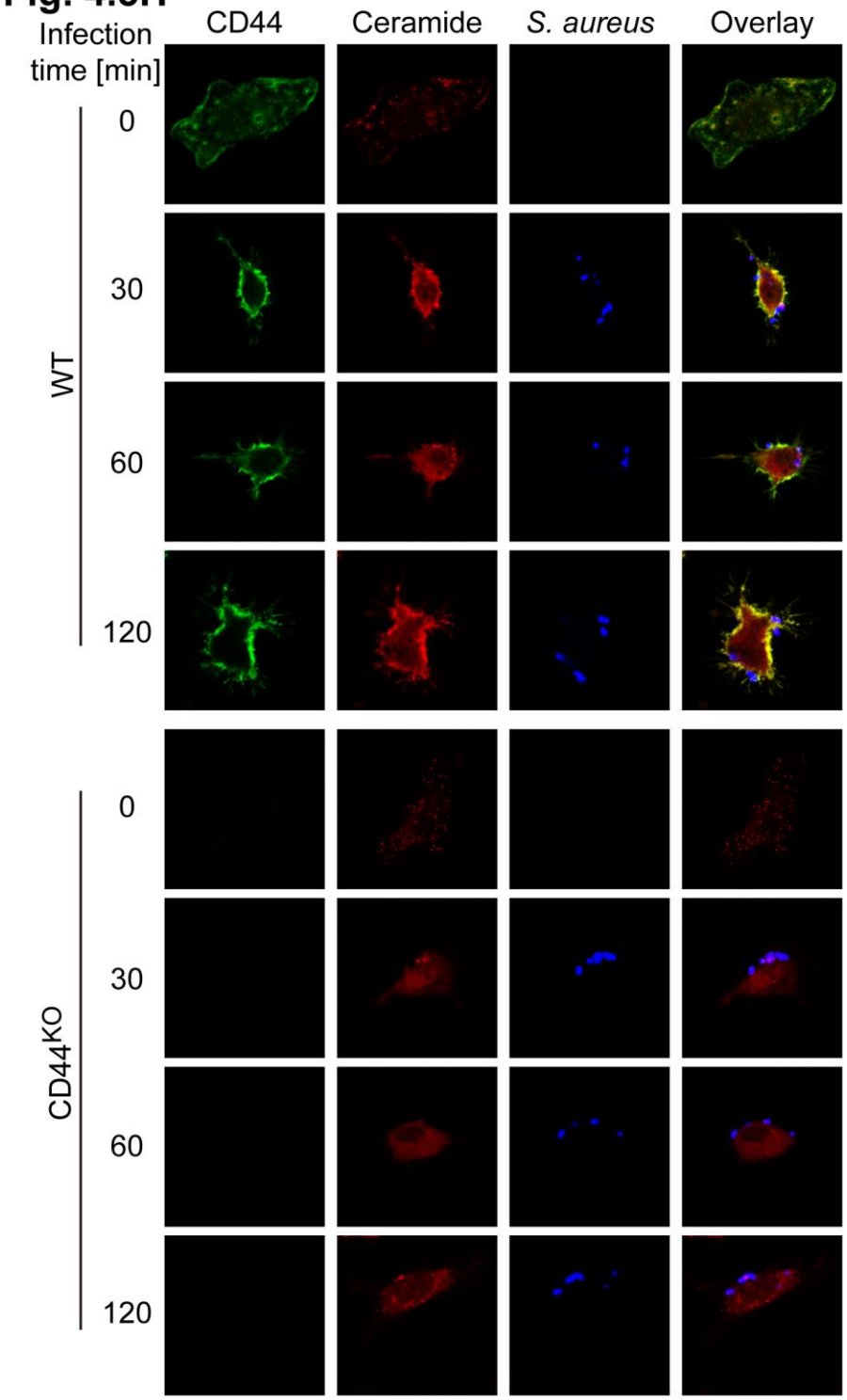

CD44
Ceramide
S. aureus
Overlay
Infection time [min]
WT
0
30
60
120
CD44KO
0
30
60
120

**Figure 4.5: Acid sphingomyelinase activation upon *S. aureus* infection requires CD44**

(A, B) Wild-type (WT) and CD44-deficient bone marrow-derived macrophages (BMDMs) were left uninfected or infected with S. aureus for 30, 60, or 120 min. Acid sphingomyelinase (Asm) activity was determined by the consumption of BODIPY-FLC12-sphingomyelin. Panel A shows representative results; panel B the mean ± SD of 3 independent studies; * $p < 0.05$, ANOVA followed by Student-Newman-Keuls test. (C, D) WT and CD44-deficient BMDMs were infected with S. aureus and the number of intracellular (C) or total (D) S. aureus was determined. Shown are the means ± SD of the number of colony forming units (CFUs) from at least 3 independent experiments; * $p < 0.05$, ANOVA followed by Student-Newman-Keuls test. (E, F) Levels of phosphorylated ezrin/radixin/moesin (pERM) were determined in BMDMs left untreated or infected with S. aureus for 30, 60, or 120 min by Western blotting using phospho-specific anti-ERM antibodies (E). Aliquots were blotted with an anti-ezrin antibody to confirm similar loading of all lanes (E). Shown are representative results from 3 independent experiments. Panel F shows the quantification of the phosphorylation of ERM by ImageJ. Results are given as mean ± SD, n=3, * $p < 0.05$, ANOVA followed by Student-Newman-Keuls test. (G) Confocal studies of the actin cytoskeleton using FITC-phalloidin and staining with Cy3-coupled anti-pERM antibodies demonstrate that CD44 is necessary for cortical rearrangement of actin filaments and pERM translocation/phosphorylation of macrophages with S. aureus. (H) Confocal microscopy studies using FITC-coupled anti-CD44 and Cy3-anti-ceramide antibodies show that CD44 clusters in ceramide-enriched membrane domains after infection. CD44 deficiency prevents the formation of ceramide-enriched membrane platforms. Panels G and H show representative results from 3 independent experiments.

### 4.6 Asm-deficient mice are highly susceptible to pulmonary *S. aureus* infections

To study the role of Asm in pulmonary *S. aureus* infections *in vivo*, we intranasally infected WT and Asm-deficient mice with *S. aureus*. Most WT mice rapidly cleared the infection, and almost all WT mice survived (Fig. 4.6A). In contrast, 80% of Asm-deficient mice died within 5 days (Fig. 4.6A). To determine bacteria killing in the lung, we again intranasally infected WT and Asm-deficient mice with *S. aureus* and determined the total number of CFUs in the lung 6 and 12 h after infection. Bacterial numbers were substantially higher in Asm-deficient mice than in WT mice after 6 h and 12 h infection (Fig. 4.6B).

The failure of Asm-deficient mice to kill *S. aureus* in the lung could be explained by the findings that *S. aureus* internalization is reduced by 60% to 80% in these mice (see above) and that Asm-deficiency reduces the release of reactive oxygen species that kill extracellular pathogens, as previously shown (Peng et al., 2015; Zhang et al., 2008). Thus, to determine whether Asm-deficient mice die by an accumulation of extracellular *S. aureus*, we intranasally infected WT and Asm-deficient mice with *S. aureus* for 6 h, sacrificed the mice, homogenized the lung tissue without compromising the cell integrity, and incubated the lung homogenates with gentamycin for 1 h. Cells are impermeable to gentamicin, at least for this short time, allowing extracellular bacteria to be killed by gentamycin without affecting intracellular bacteria. The results surprisingly showed that the number of CFUs is still higher in Asm-deficient mice than in WT mice 6 h after infection (Fig. 4.6C), a finding indicating that *S. aureus* accumulates within lung cells in Asm-deficient mice. Thus, to determine whether Asm is involved in intracellular killing of *S. aureus*, we cultured WT and Asm-deficient AMs and BMDMs and determined the killing of intracellular *S. aureus* 2 h to 8 h after infection via CFU assay. WT BMDMs and AMs efficiently killed *S. aureus* (Fig. 4.6D and E). In contrast, *S. aureus* survived and replicated in Asm-deficient macrophages (Fig. 4.6D and 6E).

These findings indicate that intracellular bacteria, although initially lower in numbers because the ability of Asm-deficient macrophages to internalize *S. aureus* is reduced, are not killed by macrophages; instead, they proliferate and finally cause severe and even lethal pneumonia.

**Fig. 6A**

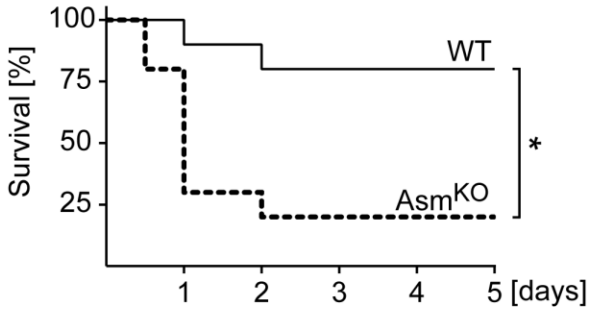



**Fig. 6B**

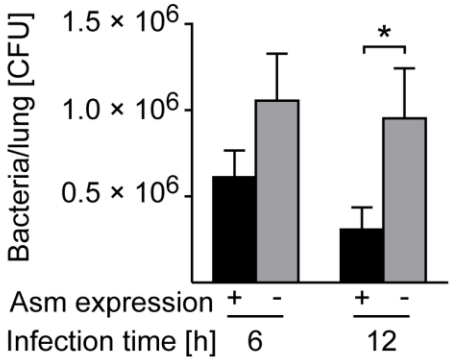



**Fig. 6C**

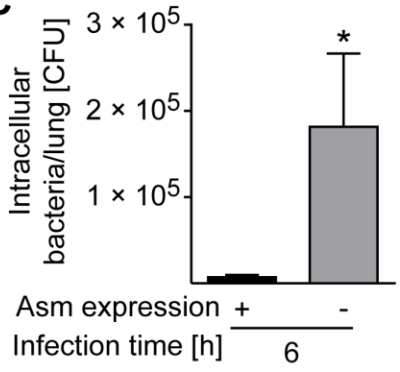



**Fig. 6D**

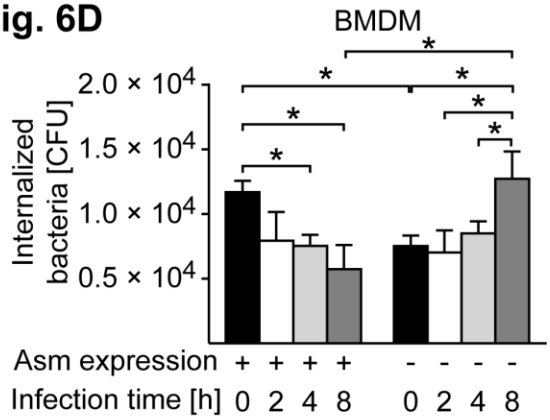



**Fig. 6E**

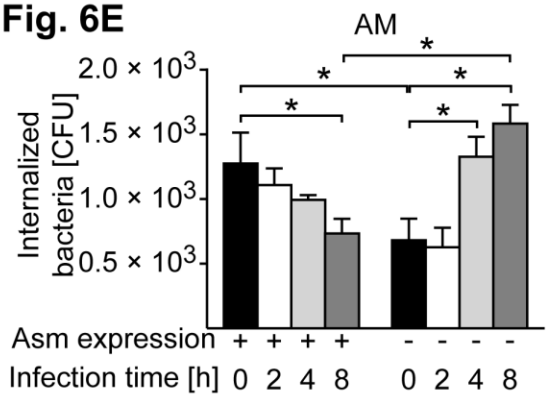


**Figure 4.6: Acid sphingomyelinase-deficient mice fail to clear *S. aureus***

**(A)** Wild-type (WT) and acid sphingomyelinase (Asm)-deficient mice were intranasally infected with $8 \times 10^8$ colony-forming units (CFUs) of *S. aureus*. Mice were observed for 5 days for survival experiments. The graph represents 10 mice from each group. Comparisons of survival variables were performed with the log-rank test. **(B)** WT and Asm-deficient mice were infected with *S. aureus*, sacrificed at 6 or 12 h after infection, lungs were homogenized and total CFU of *S. aureus* bacteria in the lung were determined. Results are shown as means ± SD; * $p < 0.05$, ANOVA followed by Student-Newman-Keuls test. **(C)** WT and Asm-deficient mice were infected with *S. aureus* for 6 h, sacrificed, lungs were homogenized and incubated with gentamycin for 1 h to kill extracellular bacteria. The numbers of bacterial CFUs is given as mean ± SD of 4 independent experiment; * $p < 0.05$, t-test. **(D, E)** Bone marrow-derived macrophages (BMDMs) and alveolar macrophages (AMs) from WT and Asm-deficient mice were exposed to *S. aureus* for the indicated times. After infection, gentamicin was added, and cells were incubated for 1 h to kill extracellular bacteria. CFU derived from lysates were determined. Data are expressed as means ± SD of 3 experiments, $p < 0.05$, ANOVA followed by Student-Newman-Keuls test.

### 4.7 Asm-deficiency leads to a failure in phagosome-lysosome fusion

To understand the mechanisms that allow intracellular *S. aureus* to survive in Asm-deficient macrophages, we investigated whether the fusion of phagosomes and lysosomes is altered in Asm-deficient macrophages. To this end, we incubated BMDMs with TMR-labeled dextran and stained the cells after fixation with antibodies to Lamp1, a lysosomal marker protein. Lamp1 staining and dextran fluorescence did not differ between WT and Asm-deficient BMDMs before infection. Instead infection with *S. aureus* induced the fusion of TMR-dextran-positive phagosomes with anti-Lamp1-labelled lysosomes in WT BMDMs (Fig. 4.7A) whereas this fusion event was abrogated by Asm-deficiency (Fig 4.7A).

We further stained the macrophages with anti-*S. aureus* antibody and Lysotracker Red, a fluorescent dye for labeling and tracking acidic organelles in living cells. *S. aureus* co-localized with Lysotracker-labeled acidic compartments in WT macrophages. Although most bacteria localized to lysosomes in WT macrophages, the fusion of phagosomes and lysosomes was defective in Asm-deficient BMDMs, and intracellular pathogens remained separated from lysosomes (Fig. 4.7B and C). In addition, staining of macrophages with Lysosensor Green, a method to measure the pH of intracellular vesicles, revealed accumulation of Lysosensor Green in intracellular compartments of WT macrophages, whereas the accumulation of Lysosensor Green was reduced in Asm-deficient macrophages (Fig. 4.7D). This indicates a failure of acidification of lysosomes in Asm-deficient macrophages upon infection.

Taken together, these findings suggest that Asm regulates phagocytosis, phagosome-lysosome fusion, and intracellular vesicle pH to mediate the killing of bacteria.

**Fig. 4.7A**

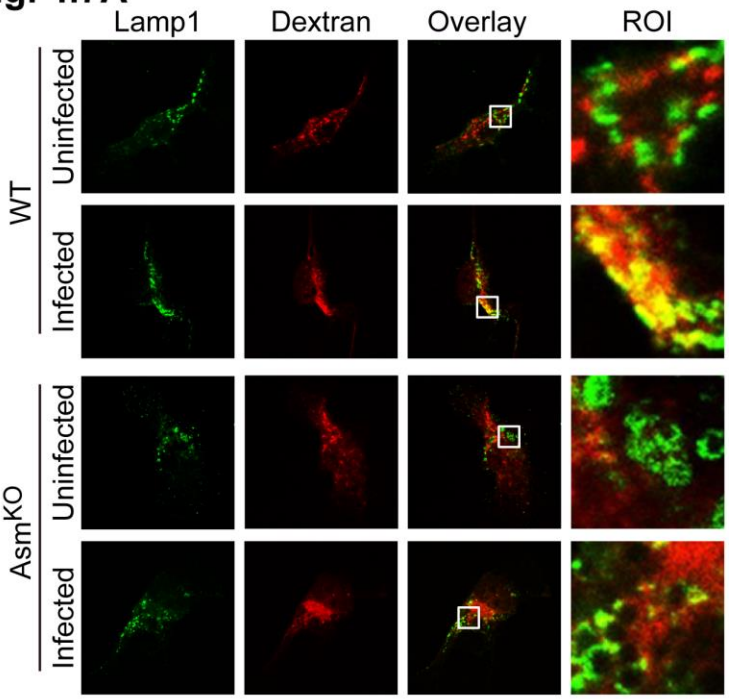



**Fig. 4.7B**

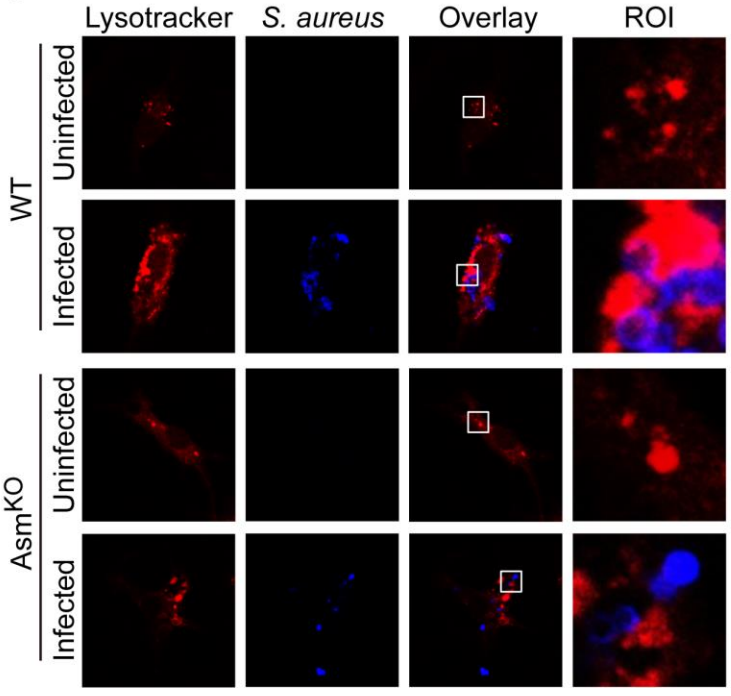



**Fig. 4.7C**

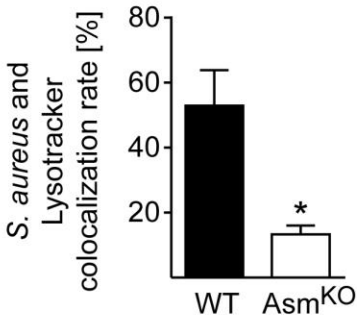



**Fig. 4.7D**

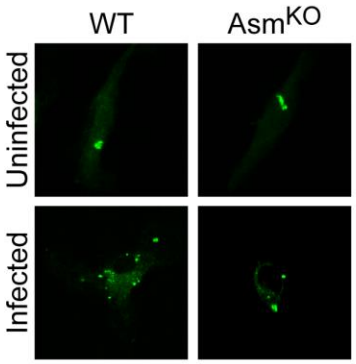


**Figure 4.7: Acid sphingomyelinase expression is required for lysosomal acidification and phagosome-lysosome fusion upon infection of macrophages with *S. aureus***

**(A)** Wild-type (WT) or Asm-deficient bone marrow-derived macrophages (BMDMs) were left untreated or were infected with S. aureus. Cells were stained with FITC-labeled anti-Lamp1 antibodies and TMR-dextran and were analyzed by fluorescence microscopy. Representative fluorescence images from four independent experiments are shown. **(B, C)** Samples were stained with LysoTracker and Cy5-labeled S. aureus for confocal microscopy studies (B). Panel C shows the measurement of the percentage of localizing bacteria as determined by LysoTracker DND-99. Results are means ± SD of 4 independent experiments; at least 100 cells were used for calculation; * $p < 0.05$, t test. **(D)** Macrophages were stained with LysoSensor DND-189 for detection of the pH of intracellular acidic compartments. Shown are representative fluorescence microscopy studies from four independent experiments.

## 5 Discussion

The present results identify CD44 as a novel macrophage receptor for *Staphylococcus. aureus* that is linked via activation of the Asm-ceramide system to internalization of the pathogen, fusion of phagosomes with lysosomes and intracellular killing of the pathogen. CD44 and Asm seem to act in a positive feedback loop with Asm activation induced by CD44 binding as an initial event that is then amplified by clustering of CD44 in ceramide-enriched membrane platforms. Additional activation of Asm by the clustered receptor and further clustering of CD44 finally lead to amplification of intracellular signaling as a positive feedback cycle. The generation of a strong signal by CD44 within a small, defined area of the cell membrane may allow the receptor to transmit the signal into macrophages, resulting in the activation of small G proteins and the rearrangement of the cytoskeleton, an event that finally mediates the uptake of *S. aureus* and intracellular killing in WT macrophages. The absence of Asm prevents this cascade of events.

### 5.1 Asm-ceramide system mediated phagocytosis of *S. aureus*

Internalization of bacteria into host cells may allow the pathogen to attack the host organism, if the pathogen can survive in the infected cells. On the other hand, internalization may also allow the host to target a pathogen to phagolysosomes, thereby killing the invading pathogen. Our lab has demonstrated Asm generated ceramide enriched platforms are essential for the internalization of *P. aeruginosa* into mammalian cells, which can be prevented by disruption of these platforms by pharmacological inhibitor or Asm gene deficiency (Grassme et al., 2003). Similar results have demonstrated the crucial role of Asm-ceramide system in non-phagocytic and phagocytic cells internalizing bacteria, which are *N. gonorrhoeae* and *N. meningitidis* (Grassme et al., 1997; Hauck et al., 2000; Simonis et al., 2014). However, the precise mechanism that how ASM-ceramide system mediates intracellular bacteria survival and replication within the host cell is poorly known.

The present studies show that the internalization of *S. aureus* is an important part of the defense against the pathogen: Asm-deficient macrophages that exhibit a reduced rate of internalization *in vivo* and *in vitro* are also unable to kill internalized *S. aureus*. Our

studies in which we used gentamycin to kill extracellular bacteria in lung homogenates of Asm-deficient and WT mice showed that *S. aureus* accumulates *in vivo* within Asm-deficient cells, because the pathogen is not killed within Asm-deficient macrophages even if these cells primarily internalize fewer bacteria. In addition, our results showed Asm-deficient mice is highly susceptible to pulmonary *S. aureus* infections *in vivo*. These findings strongly suggest that internalization and subsequent killing of the pathogen is an important part of the host defense in WT macrophages and lungs, respectively. The results of recent studies are consistent with this concept, showing that blocking the phagocytosis of *S. aureus* contributes to increased bacterial survival in human blood, bacterial persistence, and abscess formation both in pneumonia and after intravenous infection *in vivo* (Jongerius et al., 2012; Ko et al., 2013).

## 5.2 Asm-ceramide system mediated elimination of *S. aureus*

### 5.2.1 Asm and reactive oxygen species

The current study has implicated that Asm-deficient mice are highly susceptible to pulmonary *S. aureus* infections *in vitro* and *in vivo*. Multiple mechanisms can be proposed to explain the function of Asm taking into account the susceptibility. Our group has previously demonstrated that genetic deficiency of Asm abolishes the extracellular release of reactive oxygen species (ROS) after the infection of macrophages with *P. aeruginosa* (Zhang et al., 2008). In freshly isolated macrophages Asm generated ceramide enriched platforms are required for the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and release of ROS. ROS is critical components of the antimicrobial repertoire of mammalian cells (Slauch, 2011). Two subunits of NADPH oxidase $p47^{phox}$ and $gp91^{phox}$ have been shown critically involved in Asm-ceramide regulated signaling (Reinehr et al., 2006; Zhang et al., 2008). Further, we have shown Asm is activated by *S. aureus* thereby the ceramide enriched platforms are generated in endothelial cells, this signaling triggers the release of superoxide (Peng et al., 2015). However, although this mechanism may contribute to the

extracellular killing of *S. aureus* in the lung, our findings show that intracellular killing of the pathogen is also required for its elimination.

Our previous work has shown that Asm-deficient mice are more susceptible than WT mice to systemic, septic *S. aureus* infections (Peng et al., 2015). These studies demonstrated that systemic infection with *S. aureus* destroys tight junctions of endothelial cells in the lung, thereby inducing lung edema. Whether destruction of endothelial tight junctions is mediated by intra- or extracellular *S. aureus* or toxins is presently unknown.

### 5.2.2 Asm and phagosome-lysosome fusion

Our findings have revealed a mechanism that deficiency of Asm allows the survival and replication of intracellular *S. aureus* in macrophages, which is Asm-deficiency leads to a failure in phagosome-lysosome fusion. No difference of Lamp1-staining and dextran fluorescence between WT and Asm-deficient BMDMs before infection indicates that Asm deficiency does not lead a phenotype, whereas the fusion of TMR-dextran-positive phagosomes with anti-Lamp1-labelled lysosomes is blocked by Asm-deficiency after infection of *S. aureus*. Furthermore, internalized *S. aureus* localized to lysosomes in WT macrophages but not in Asm deficient macrophages. In addition, Asm deficiency reduces the accumulation of Lysosensor Green marked intracellular acidic compartments. All of these findings implicates that Asm-ceramide system regulates the phagocytosis by fusion of phagosome with lysosome and acidification of intracellular vesicles. Most bacteria are rapidly eliminated and degraded in the phagolysosome, thus, the failure of bioprocess of phagolysosome formation by Asm deficiency leads to the intracellular bacteria survival.

It has been demonstrated that Asm deficiency highly impairs the bactericidal capacity of mice challenging the *L. monocytogenes*, which due to the fail of macrophages intracellular bacteria killing (Utermohlen et al., 2003). The dose of LD50 with *L. monocytogenes* infection for Asm deficient mice was 100 times lower than WT mice, an

effect may account from reduced intracellular listeriocidal activity in Asm deficient macrophages. Further the same group extended the study and demonstrated the Asm is required for efficient phago-lysosomal fusion in *L. monocytogenes* infection (Schramm et al., 2008). *L. monocytogenes* rapidly escape from phagosome into the cytosol in Asm deficient macrophages. The Asm deficiency reduces the co-localization of intracellular *L. monocytogenes* with the late endosome/lysosome marker Lamp1. The mechanism is that ASM does not impair the maturation of phagosome in the early stage but delays fusion of lysosomes with phagosomes in the late stage in macrophages. In Asm deficient macrophages, the existence of listeriocidal proteases cathepsin D, B and L in *L. monocytogenes* contained phagosomes significantly decreased upon infection. Asm generated ceramide targets and enhances the activation of the lysosomal cathepsin D (Heinrich et al., 1999).

Recent studies have shown Asm interacting with proneurotrophin receptor sortilin mediates the infection process of *M. tuberculosis* in macrophages (Vazquez et al., 2016). Sortilin mediates the Asm trafficking from Golgi complex into mycobacterial containing phagosomes. Once delivered to phagosome, Asm localizing with lysosomal-associated membrane protein (Lamp) 2 serves to the growth restriction and elimination of *M. tuberculosis* in bone marrow derived macrophages. Moreover, depleting ASM by a pharmacological inhibitor desipramine increases the survival of *M. tuberculosis*. Similar with this notion, addition of ceramide is found facilitating the clearance of *M. tuberculosis* and *M. avium*, by significantly increasing the fraction of acidified phagosomes in macrophages (Anes et al., 2003).

Thus it is very likely Asm regulate the killing of *S. aureus* by host cells via modulating the bioprocess of phagocytosis, in particular the fusion and maturation of phagolysosome.

### 5.2.3 Asm and cytokine and chemokine release

Asm-ceramide system has been demonstrated in regulation of cytokines and chemokines release upon several bacteria. When exposed to bacterial infection,

macrophage secretes inflammatory cytokines such as TNF-α and IL-1β, and chemokines which drive an immune reaction (Turner et al., 2014). Abnormities of cytokine and chemokine release is involved in multiple infectious diseases.

Our group has demonstrated that *P. aeruginosa* infection leads to the increased mRNA transcription of IL-1β as well as uncontrolled released of IL-1β from infected cells or lungs of Asm deficiency (Grassme et al., 2003). Adding of exogenous ceramide is sufficient to rescue the uncontrolled release of IL-1β in Asm deficient epithelial cells. More studies has implicated blocking of Asm with a multiple approaches, i.e. silencing of Asm, pharmacological Asm inhibitor, or adding Asm antibody significantly increased the IL-8 release with *P. aeruginosa* infection in epithelial cells (Yu et al., 2009).

Asm can involve in the bacterial toxin induced cytokine and chemokine release, such as LPS. Inhibiting of NF-kB pathway by a cell penetrating peptide sufficiently suppresses the Asm activation upon LPS stiumulation. The ceramide mediated production of TNF-α, IL-6, CXC chemokine CXCL8, and MCP-1, these key regulators of inflammation are also found reduced by NF-kB inhibition (von Bismarck et al., 2012). Studies also show the effect of LPS on Asm activation is involving the production of IL-1β (Wong et al., 2000). This is similar that LPS leads to the production of TNF-α and other cytokines which stimulates the hydrolysis of sphingomyelin to ceramide by Asm (Haimovitz-Friedman et al., 1997). Vice versa, the Asm activation by LPS is required for the release of TNF-α (Cuschieri et al., 2007).

**5. 3 Asm activation and CD44**

Previous studies have shown that the Asm-ceramide system is involved in infection with several pathogenic bacteria, such as *Neisseria gonorrhoeae*, *N. meningitides*, *Pseudomonas aeruginosa*, *S. aureus*, and *Salmonella species* (Hauck et al., 2000; McCollister et al., 2007; Peng et al., 2015; Simonis et al., 2014; Zhang et al., 2008). However, the molecular mechanisms of Asm activation by bacteria are still poorly

understood. Our current studies identify for the first time a receptor, i.e., CD44 that couples a pathogen with activation of Asm.

Our results has shown CD44 and Asm seem to act in a positive feedback loop with Asm activation induced by CD44 binding as an initial event that is then amplified by clustering of CD44 in ceramide-enriched membrane platforms. Recent studies have shown hyaluronan tetrasaccharides, the smallest unit of hyaluronan, bind to its receptor CD44; thereby stimulate ceramide production through upregulated mRNA expression of Asm (Kage and Tokudome, 2016).

Our findings do not show a complete absence of Asm activation, ceramide formation, cytoskeletal changes, and internalization in CD44-deficient cells. This result may be explained by the binding of the pathogen to additional receptors, such as intercellular adhesion molecule 1 (ICAM-1), that may also couple with Asm (Olaku et al., 2011). Cells lacking Asm activity was found an increased expression of ICAM-1 (Lopes Pinheiro et al., 2016). Asm-ceramide system coordinating with ICAM-1 functions in T cell transmigration.

Similarly, Asm deficiency does not completely abrogate the internalization of *S. aureus*. Because cells contain only a single Asm gene, it may be possible that other pathways independent of Asm are also involved in the uptake of the pathogen.

Asm hydrolyzes sphingomyelin to ceramide, which has been shown to spontaneously form ceramide-enriched platforms in the plasma membrane (Grassme et al., 2001a). These platforms trap and cluster specific proteins, thereby inducing and amplifying signaling transduction (Bock and Gulbins, 2003; Grassme et al., 2001b). This mechanism may also apply to the positive feedback between CD44 and Asm activation. CD44 consists of three regions: an extracellular domain, a transmembrane domain, and a C-terminal cytoplasmic domain (Goodison et al., 1999). The results of structural studies of CD40 indicated that the transmembranous domain of CD40 determines clustering within ceramide-enriched membrane domains (Bock and Gulbins, 2003). It is tempting to speculate that a similar mechanism applies to CD44, although the exact

mechanisms by which receptors cluster in ceramide-enriched membrane domains are unknown.

### 5. 4 Asm and cytoskeleton reorganization

Our findings have demonstrated Asm mediates the phosphorylation of ERM proteins in the infection process of *S. aureus*. Asm deficiency reduces the co-localization of phosphorylated ERM (pERM) with phalloidin and filopodia. Additionally, Asm deficiency abolished the activation of Rho GTPase RhoA, Rac1, and Cdc42 upon *S. aureus* infection.

#### 5.4.1 Asm and ezrin/radixin/moesin (ERM) proteins

ERM proteins have been implicated as critical organizers of actin dynamics (Fehon et al., 2010; Tsukita and Yonemura, 1999). ERM proteins are able to interact with transmembrane proteins such as CD44, lipids, membrane associated cytoplasmic proteins and cytoskeleton. Phosphorylation of ERM proteins leads to their structure conformational change and activation. Our group recently observed that exogenous adding of Asm resulted in phosphorylation of ezrin in B16F10 or human melanoma cells (Carpinteiro et al., 2016). However, the phosphorylation of ezrin also was found after the co-incubation of B16F10 melanoma cells with Asm-deficient platelets, which indicates that ezrin phosphorylation can be induced not only Asm but also different pathway independent of this enzyme. The author proposed a mechanism that both exogenous adding of Asm (Canals et al., 2010) and physiological contact of platelets (Yatomi et al., 1995) with tumor cells induces the generation of sphingosine-1-phosphate (S1P). Therefore, S1P then leads to the phosphorylation of ezrin (Canals et al., 2010).

Similar to our result, a study has shown Asm is required for the phosphorylation of ezrin and formation of microvilli, ultimately induce T cell mobility and transmigration (Lopes Pinheiro et al., 2016). Furthermore, ERM proteins were also less phosphorylated upon ICAM-1 clustering in Asm-deficient cells.

Interestingly, other studies have also implicated that phosphorylation of ERM proteins can be regulated by ASM-ceramide system (Zeidan et al., 2008a). In that study, chemotherapeutic agent cisplatin leads to an activation of Asm activity and translocation of Asm to the cell membrane, which induces dephosphorylation of ezrin and loss of lamellipodia/filopodia in breast cancer cells. Reconstitution of Asm or exogenous delivery of ceramide recapitulates the morphotropic effects of cisplatin. Collectively, these results indicate a suppressor characteristic of Asm-ceramide system in ERM protein phosphorylation.

Our result shows a marked increase of pERM proteins upon *S. aureus* infection in macropahges. The discrepancy may be because of the type of cells used. Macrophages are phagocyte and serve for pathogen killing in our study whereas cancer cells are highly proliferative in comparison with macrophages. Further, we studied specific bacteria-host interaction as opposed to cancer cell migration, these interaction are more individual and related to their specialized function.

Further, ERM proteins can be activated and phosphorylated to a wide variety of signaling pathways, for example, protein kinase C (PKC) (Ng et al., 2001). This would be a potential mechanism in Asm-ceramide induced ERM phosphorylation upon *S. aureus* infection. Asm-ceramide system has been shown plays an important role in regulating activation of PKC isoforms in a variety of studies (Cuschieri et al., 2007; Gilbert et al., 2016; Kasai and Tanabe, 2014; Parent et al., 2011).

The precise mechanism regarding how exactly ERM phosphorylation is controlled and in what cellular and developmental contexts is regulated by Asm-ceramide system remains to be elucidated. Detecting the precipitation of ceramide with ERM proteins would be strong evidence which confirms the interaction this signaling in the infection process of *S. aureus*.

### 5.4.2 Asm and Rho GTPase

Our studies have demonstrated Asm is critically required for the activation of Rho GTPase upon *S. aureus* infection. The Rho family of GTPase is a subfamily of Ras superfamily and consists of 20 members in human, of which Rho, Rac and Cdc42 remain the best studied (Hodge and Ridley, 2016; Jaffe and Hall, 2005). Once activated, Rho GTPases bind to a variety of effectors including protein kinases and some actin-binding proteins, regulate cytoskeletal and cell adhesion dynamics and thereby coordinate a wide range of cellular processes.

The association of Rho GTPase with plasma membranes is modified by lipids (Hodge and Ridley, 2016). The G domain of Rho GTPase is highly conserved whereas the C terminal contains a hypervariable motif. Rho GTPase is specified through the hypervariable domain at the carboxyl terminus which contains several important sequences. For example, the CAAX motif of RhoA at C terminus is modified by a variety of post-translational lipid modifications including farnesylation, geranylgeranylation and palmitoylation. These lipid moiety connect the Rho GTPase to the cell membrane avoids diffusing through the cytoplasm. Thus, the Rho GTPase can localize to distinct membrane compartments on lipid bilayer. However, the existence of lipid rafts or similar membrane domains in the inner leaflet of cell membrane is not known currently. Resolving how cholesterol and sphingolipid dependent lipids rafts interact with Rho GTPase promises to be a difficult but rewarding undertaking.

It has been shown that depletion of sphingolipids decreases targeting of RhoA and Cdc42 to the cell membrane, a process which could be partially recovered by exogenous adding of sphingomyelin (Cheng et al., 2006). Sphingomyelin is critically required for *in vivo* membrane targeting and *in vitro* binding to artificial lipid vesicles of RhoA and Cdc42. This study may suggest sphingomyelin, a component of lipid rafts, plays important in Rho GTPase signaling. In addition, RhoA bounding to cell membrane is found significant reduced in Asm deficient cell, which in term impairs function of membrane remodeling.

### 5.5 CD44 and *S. aureus* infection

In several cases, CD44 isoforms were shown to participate in the host infections (Gunthert et al., 1991). CD44v6 was for example shown to promote the internalization of the food-born pathogen *Lysteria monocytogenes* upon binding of the virulence factor Internalin B to the receptor tyrosine kinase MET (Jung et al., 2009). CD44 was also described to interact with IpaB, a protein secreted by *Shigella* (Skoudy et al., 2000). The formation of this complex appeared important for invasion of epithelial cells. Later on, the interaction between CD44 and IpaB was described to take place within lipid rafts thereby promoting infection (Lafont et al., 2002). More recently peptides targeting CD44 were shown to block *Helicobacter pylori*-induced proliferation and subsequent gastritis (Bertaux-Skeirik et al., 2015). A collaboration between CD44 and MET was required in that case. Whether similar mechanisms link CD44 to the Asm remains to be determined.

Taken together, our studies describe a novel mechanism by which *S. aureus* infects macrophages: Binding of the pathogen to CD44 on macrophages activates the Asm-ceramide system, resulting in the formation of ceramide-enriched membrane platforms that in turn cluster and amplify CD44 signaling and thereby act as a positive feedback loop. CD44 and Asm are crucially involved in the activation of small G-proteins, the phosphorylation of ERM proteins, and the re-arrangement of the cytoskeleton, culminating in the phagocytosis of *S. aureus*. Phagosomes containing the pathogen fuse with acidified lysosomes in an Asm-dependent manner to kill intracellular pathogens, which is a requirement for successful host defense in the lungs. Asm deficiency reduces or prevents all of these events, allowing the intracellular survival of internalized pathogens.

## 6 Summary

*Staphylococcus aureus* is a very common commensal opportunistic bacterium that causes severe and life-threatening diseases such as pneumonia, endocarditis, sepsis, osteomyelitis, and toxic shock syndrome. In addition, some *S. aureus* strains have developed resistance to almost all antibiotics. Thus, *S. aureus* infections are a major clinical problem and mechanisms that mediate infection with *S. aureus* need to be identified to facilitate the development of novel treatments. Here, we demonstrate that CD44 serves as a receptor for *S. aureus* in macrophages. CD44 activates the acid sphingomyelinase upon infection and triggers the release of ceramide. Ceramide forms distinct domains in the plasma membrane that serve to cluster CD44 and thereby

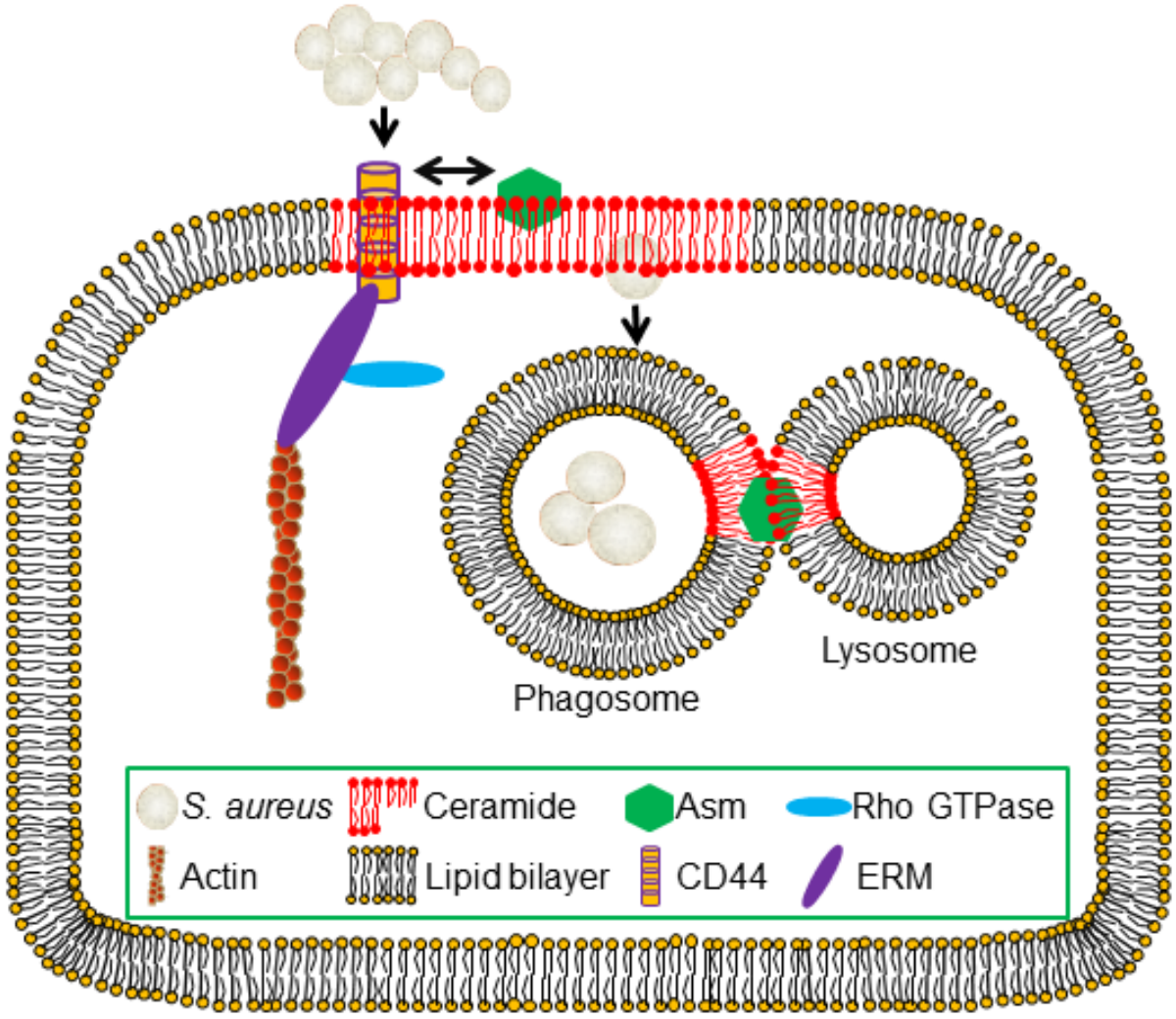



Figure 6 Asm-ceramide system regulates *S. aureus* infections

amplify CD44 signaling, which results in activation of small Rho family GTPases, reorganization of the actin cytoskeleton, internalization of *S. aureus* by macrophages, fusion of phagosomes with lysosomes and intracellular killing of the pathogen. Genetic deficiency of CD44 or acid sphingomyelinase abrogates these events. Accordingly, acid sphingomyelinase-deficient macrophages fail to kill intracellular *S. aureus* and are highly susceptible to pulmonary *S. aureus* infections. Thus, our data identify an important role of the CD44-Asm-ceramide system in the infection of macrophages with *S. aureus*.

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## Publications, Posters and Presentations

### Publications

Peng H, **Li C**, Kadow S, Henry BD, Steinmann J, Becker KA, Riehle A, Beckmann N, Wilker B, Li PL, Pritts T, Edwards MJ, Zhang Y, Gulbins E, Grassmé H. Acid sphingomyelinase inhibition protects mice from lung edema and lethal *Staphylococcus aureus* sepsis. J Mol Med (Berl), 2015

**Li C**, Peng H, Japtok L, Seitz A, Riehle A, Wilker B, Soddemann M, Kleuser B, Edwards M, Lammas D, Zhang Y, Gulbins E, Grassme H. Inhibition of neutral sphingomyelinase protects mice against systemic tuberculosis. Front Biosci (Elite Ed), 2016

Fahsel B, Kemper H, Mayeres J, **Li C**, Wilker B, Keitsch S, Kohnen M, Caldwell C, Fraunholz M, Edwards M, Grassmé H, Gulbins E, Seitz A. *Staphylococcus aureus* alpha-toxin disrupts endothelial tight junctions via acid sphingomyelinase, ceramide and p38K, In press, 2016

**Li C**, Wu Y, Riehle A, Kamler M, Gulbins E, Grassmé H. *Staphylococcus aureus* survives in cystic fibrosis macrophages forming a reservoir for chronic pneumonia. Infect & Imm, in revision, 2016

**Li C**, Wu Y, Riehle A, Orian-Rousseau V, Gulbins E, Grassmé H. Regulation of *Staphylococcus aureus* infection of macrophages by CD44 and acid sphingomyelinase. Plos Pathogens, submitted, 2016

**Li C**, Gulbins E, Grassmé H. Acid sphingomyelinase in host bacteria interaction. In submission, 2016

**Posters and Presentations**

**Li C**, Gulbins E, Grassmé H. Regulation of the cytoskeleton by ceramide upon *Staphylococcus aureus* infection. Poster, Tag der Forschung der Medizinischen Fakultät, Essen, Nov. 21, 2014

**Li C**, Gulbins E, Grassmé H. Regulation of the cytoskeleton by ceramide upon *Staphylococcus aureus* infection. Poster and Presentation, 3rd International Meeting of German Society for Cell Biology on Actin Dynamics, Regensburg, Germany, May 2-5, 2015

**Li C**, Gulbins E, Grassmé H. Regulation of the cytoskeleton by ceramide upon *Staphylococcus aureus infection*. Poster, Sphingolipids in infection and beyond, Wuerzburg, Germany, June 25-26, 2015

**Li C**, Gulbins E, Grassmé H. Acid sphingomyelinase regulates infection of macrophages with *Staphylococcus aureus* - Application to the pathogenesis of cystic fibrosis. Poster, Tag der Forschung der Medizinischen Fakultät, Essen, Nov. 20, 2015

**Li C**, Wu Y, Gulbins E, Grassmé H Inhibition of neutral sphingomyelinase protects mice against systemic tuberculosis. Presentation, Forschergruppen Meeting (FOR2123), Wuerzburg, Germany, Feb. 4, 2016

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